UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIA

Trends in seahorse abundance in the Ria Formosa, South Portugal: recent scenario and future prospects

Miguel José Teodoro Correia

Tese para obtenção do grau de Doutor
em Ciências do Mar, da Terra e do Ambiente
(Ramo Ciências do Mar)

Trabalho efectuado sob a orientação de:
Professor Doutor José Pedro Andrade, Universidade do Algarve
Doutora Heather Koldewey, ZSL

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Este trabalho foi realizado no âmbito da tese de Doutoramento de Miguel Correia

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Assinado:

(Miguel Correia)

RESUMO

Durante a década anterior, a investigação desenvolvida sobre os cavalos marinhos da Ria Formosa, o cavalo marinho de focinho comprido (*Hippocampus guttulatus*) e o cavalo marinho de focinho curto (*H. hippocampus*) permitiu identificar populações relativamente estáveis e, simultaneamente, a maior densidade de efetivos destas duas espécies, a nível mundial. Contudo, observações mais recentes revelaram em inúmeros locais da Ria, um decréscimo muito significativo destes efetivos, que atingiu 94% em *H. guttulatus* e 73% em *H. hippocampus*. Perante este resultado, tornou-se imprescindível e urgente não só investigar as causas que determinaram a variação da abundância destas populações de cavalos marinhos, através de ações de monitorização mensais, mas também (e igualmente importante) desenvolver ferramentas que possam responder positivamente ao restabelecimento do níveis de abundância de cavalos marinhos na Ria Formosa.

De modo a verificar a situação atual das populações de cavalos marinhos e identificar a sua dinâmica populacional, foram realizados censos em vários locais de amostragem, já previamente monitorizados em anos anteriores, i.e. 2001/2002 e 2008/2009 permitindo dessa forma estabelecer uma comparação histórica da informação. Após a comparação dos resultados estes revelaram uma aparente recuperação das populações da espécie *H. guttulatus* desde 2008/2009 mas ainda em valores inferiores aos observados em 2001/2002. De todas as variáveis testadas, foi apenas possível estabelecer uma correlação significativa entre a percentagem de coberto de fundo e a densidade de *H. guttulatus* ($p<0.001$). Os modelos de correlação utilizados (Modelos Lineares Generalizados Mistos - GLMM) encontraram uma correlação positiva entre densidades de *H. guttulatus* e *H. hippocampus*. Estes resultados realçam a importância da presença de estruturas de fixação na abundância de cavalos marinhos, no entanto poderão existir outras variáveis não testadas neste estudo que poderão contribuir para as flutuações da abundância das populações de cavalos marinhos. Assim sendo, é aconselhável manter um plano de monitorizações de modo a encontrar outras variáveis/causas para estas flutuações.

De modo a otimizar a técnica de amostragem nas campanhas de monitorização mensal, desenvolveu-se uma nova metodologia de monitorização subaquática que, comparada com a

anteriormente utilizada provou ser mais eficaz e representativa da abundância de cavalos marinhos na área estudada. A metodologia anterior baseava-se no uso de três transetos de 30 metros cada dispostos de uma forma aleatória de modo a cobrir uma área de amostragem de 180 m^2. A nova metodologia proposta sugere o uso de dois transetos de 30 metros cada, dispostos em paralelo e distando 4 metros entre si, possibilitando assim a monitorização de uma mesma área nos vários momentos de amostragem, cobrindo uma área total de 240 m^2. Os resultados obtidos nesta experiência evidenciaram a importância do uso de uma metodologia adequada ao local de estudo. Zonas com pouca abundância ou com estruturas de fixação dispersas estão sujeitas a uma subestimação do valor real de abundância da população de cavalos marinhos caso a metodologia anterior seja aplicada. No entanto, o novo método de monitorização provou ser mais representativo do local de estudo já que foram observados um maior número de indivíduos por área. Esta metodologia agora proposta poderá ser usada em situações semelhantes, noutros locais e mesmo noutras espécies.

Tendo em conta as flutuações encontradas no presente estudo, foram selecionados 6 locais com presença de cavalos marinhos e que diferiam entre si em fatores como profundidade, tipo de substrato, abundância de estruturas de fixação, hidrodinamismo e pressão de atividades humanas (pesca, tráfego de barcos, atividades de lazer). Estes locais foram monitorizados mensalmente entre Janeiro e Dezembro de 2012. No decurso deste estudo, foram identificadas variações nas densidades de cavalos marinhos nos diferentes locais escolhidos, com provável causa em fatores de stress inerentes a atividades antrópicas (pesca, tráfego marítimo e dragagens) ou mesmo a eventos naturais como o assoreamento, bem como a disponibilidade de estruturas de fixação, essenciais para a permanência dos cavalos marinhos numa determinada área. Procedeu-se igualmente ao estudo comparativo das abundâncias nos diversos anos de amostragem, Os resultados obtidos indicam uma correlação positiva entre a presença de estruturas de fixação e densidade de cavalos marinhos. Por outro lado, as observações *in situ* revelaram a presença de um número importante de juvenis, o que se apresenta como um dado promissor para a recuperação destas populações de cavalos marinhos na Ria Formosa.

Adicionalmente, desenvolveu-se e aplicou-se uma nova técnica para estudos de abundância da espécie *H. guttulatus*, de forma a se poder fazer a monotorização e identificação individual

dos efetivos populacionais sem recurso a técnicas invasivas. Esta técnica apresentada provou ser uma ferramenta viável e não invasiva quando comparada com outras mais comummente utilizadas (e.g. Visual Implant Fluorescent Elastomer – Tinta fluorescente injetada). Ao verificar-se que a espécie *H. guttulatus* possui um padrão de pintas aparentemente único para cada indivíduo, essa hipótese foi inicialmente testada em cativeiro em indivíduos adultos e juvenis (6 meses de idade). O método consistiu em tirar uma fotografia da face esquerda dos cavalos marinhos e posteriormente analisar a fotografia num programa informático desenvolvido para estudos semelhantes (programa Manta I3SM v2.1). Em face dos bons resultados obtidos, que permitiram uma comparação positiva numa elevada percentagem dos casos (>80%), esta técnica foi então efetuada nas monitorizações mensais de modo a poder estimar a abundância dos locais bem como identificar padrões de movimentos. Também ai a taxa de re-avistamento foi significativa. Esta ferramenta tem um enorme potencial de utilização não só para a espécie *H. guttulatus* mas também para todas as restantes espécies do género *Hippocampus* que possuam padrões corporais identificativos.

Para além da monotorização sazonal e avaliação de técnicas eficazes a utilizar para esse propósito, pretendeu-se também encontrar respostas para a gestão e conservação das populações de cavalos marinhos na Ria Formosa. Assim, para esse efeito, foram desenvolvidas unidades artificiais de abrigo (UAA) com o objetivo de verificar a sua utilidade na recuperação de áreas impactadas e em risco de degradação ambiental, mas em que apesar de tudo tivessem sido registadas agregados populacionais de cavalos marinhos. Inicialmente diferentes estruturas foram testadas em laboratório de modo a avaliar a preferência dos cavalos marinhos. Tendo em conta os padrões de relação da espécie *H. guttulatus* com o coberto de fundo foram testadas quatro diferentes estruturas de fixação (uma simulação de pradarias marinhas (Zostera sp.), duas simulações da macroalga *Codium* sp. de diferentes grossuras e uma quarta simulação de uma estruturas artificial rígida) em diferentes densidades e sob diferentes intensidades de corrente. Após se verificar uma maior propensão por parte dos cavalos marinhos para a utilização de estruturas de fixação artificiais semelhantes à macroalga *Codium* sp., estas foram utilizadas na construção de UAA e colocadas em ambiente natural, inicialmente através de um protótipo e depois colocada em 4 locais na Ria Formosa, dispostas em triplicados, com uma área total de cobertura por local de $12m^2$. Estes locais diferiam entre si em fatores como a profundidade, hidrodinamismo e proximidade a

populações de cavalos marinhos estabelecidas. Os resultados obtidos revelaram que todas as UAA foram prontamente colonizadas, observando-se em todas elas densidades de utilização quase sempre superiores ao observado no ambiente circundante, tendo-se mesmo atingido num dos locais, cerca de 13 cavalos marinhos.m^2, valor muito acima dos reportados em estudos anteriores.

Por outro lado, as estruturas aparentaram ter uma maior relevância quando colocadas em zonas de menor complexidade de habitat, já que em locais com maior presença de estruturas de fixação (naturais ou artificiais), os cavalos marinhos preferem manter-se nessas mesmas áreas em detrimento das UAA colocadas. Num dos locais selecionados, verificou-se, um evento de assoreamento progressivo num dos locais selecionados, resultando no desaparecimento das UAA, que ficaram cobertas com areia e assim, inutilizadas. Este facto sugere que estas UAA não poderão ser colocadas em zonas de migração de barras, já que se tratam de zonas propensas a eventos de assoreamento e por isso limita a ação de estruturas artificiais. Os resultados obtidos neste estudo atestam a viabilidade do uso das UAA como ferramentas de recuperação de habitat, possibilitando o uso das mesmas em zonas degradadas.

Assim, enquadrando as diferentes experiências efectuadas neste trabalho, foi possível concluir que só através da monotorização regular das populações de cavalos marinhos da Ria Formosa num horizonte temporal alargado é possível aferir sobre a flutuações populacionais destas espécies ao mesmo tempo que as técnicas nela empregues devem ser ajustadas consoante as espécies e a sua localização. As duas espécies de cavalos marinhos existentes na Ria Formosa apresentam diferentes requisitos ambientais, requisitos esse que condicionam grandemente a sua abundância. A maior dependência do cavalo marinho de focinho comprido para com ambientes de maior complexidade podem também torná-lo mais vulnerável às alterações ambientais de origem natural e/ou antropogénica, agora parcialmente identificadas. Finalmente, foi possível confirmar a utilidade de UAA como uma ferramenta válida para a mitigação e recuperação de habitats degradados e por isso, poderão ser utilizadas em futuros planos de gestão e conservação das populações de cavalos marinhos da Ria Formosa.

Palavras-chave: *Hippocampus guttulatus*; *Hippocampus hippocampus*; monitorização subaquática; foto-identificação; estruturas artificiais.

ABSTRACT

In the last decade, seahorse population census in the Ria Formosa identified stable populations of *Hippocampus guttulatus* and *H. hippocampus* at the highest densities described for these species worldwide. However, recent studies in the Ria reported a significant decrease in seahorse numbers of 94% for *H. guttulatus* and 73% for *H. hippocampus*.

Therefore, this study aimed to identify aspects responsible for the variation in seahorse abundance, first by looking at the current trend of seahorse population by comparing it with previous surveys. Secondly, new techniques were tested to improve the survey efficiency of seahorse population and new tools were developed to promote seahorse population recovery, with potential application in seahorse population management programs.

In this study, it was observed that both seahorse species abundance increased since the previous survey (2008/2009) although at lower numbers than in the 2001/2002 surveys and a significant correlation between the percentage coverage and *H. guttulatus* density was found. The new underwater visual census tested, proved to be more effective and accurate in a low seahorse density scenario. In this study, seasonal variations in seahorse density were observed in all sites and were probably related to human activities, natural events, and holdfast availability. In addition, a new photo-identification method was tested and results indicate it as a suitable tool for capture-recapture studies and less invasive that other commonly used methods. To cope with habitat degradation, artificial holdfast units (AHU) were designed and first tested in laboratory to assess seahorse preference. The preferred AHU was then deployed in 4 locations in the Ria Formosa, that differed in depth, water current and seahorse abundance in the vicinities of the selected site. Results showed seahorse settlement at high densities and validated the usefulness of the AHU as a habitat recovery tool.

Key-words: *Hippocampus guttulatus*; *Hippocampus hippocampus*; underwater visual census; photo-identification; artificial structures.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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Que o futuro seja tudo o que dele quiseres.

"*What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on." - Jacques Yves Cousteau*

Chapter 1. General introduction

In the past century, much attention has been focused in population biology and the debate around why populations fluctuate (Moran, 1953; May, 1974; Beddington & May, 1977; Kareiva, 1987). This question continues to be contemporary and relevant, particularly due to its implications in the management and conservation of natural resources (Clark, 2010). Determining sources of variation in population abundance and identifying the factors causing those fluctuations are crucial questions in ecology (Hunter Jr & Gibbs, 2009). The main issue comes from the natural heterogeneity of ecosystems (Kolasa *et al.*, 1991). Therefore, studies on spatial and temporal variability of unexploited species, as well as the environmental and biological factors driving this variability are urgently required (Martin-Smith & Vincent, 2005).

Marine fish communities are influenced by biotic (competition, predation, reproduction, etc.) and environmental factors (temperature, salinity, habitat complexity, depth, etc.) (Baillie *et al.*, 2004). These factors have been identified as major role players in controlling the structure of these communities in the near-shore environment i.e, abundance, spatial distribution and species diversity (Thrush & Dayton, 2002; De Raedemaecker *et al.*, 2010). Baillie *et al.* (2004) reported that most marine extinctions were attributed to exploitation (55%) and habitat loss/degradation (37%), nevertheless, the effects of habitat loss are difficult to separate from other impacts as they are commonly combined. Human induced habitat loss has been caused by coastal development with associated pollution, leading to sediment loading and added nutrients inputs (Short & Wyllie-Echeverria, 1996). Anthropogenic pressure due to tourism and illegal fisheries, amongst others, have a negative impact on the ecosystems as it alters the habitat structure of the local fish communities (Guidetti & Boero, 2004; Pinnegar & Polunin, 2004; Kucuksezgin *et al.*, 2006; Curtis *et al.*, 2007). In marine benthic habitats, small-scale natural disturbances can lead to populations' patchiness. Many these disturbances that impact benthic communities can promote spatial heterogeneity with implications for the maintenance of diversity and stability at the local populations (Thrush & Dayton, 2002). *In situ* observation presents many advantages in order to assess the impact of anthropogenic disturbances on the structure of benthic communities, when compared to

experimental methods (Warwick *et al.*, 1990). Most benthic organisms can encompass environmental conditions better than pelagic due to their sessile nature (Gamito, 2006).

Habitat destruction is considered the most important driver of species extinction worldwide (Pimm & Raven, 2000). Although the maximum extinctions would only occur over a period decades due to slow habitat degradation, efforts should be done to protect and minimize damage in the biodiversity hotspots to prevent this cumulative destruction resulting in extinction. In fact only recently researchers are uncovering this event on a regional and global scale (Jackson *et al.*, 2001b; Dulvy *et al.*, 2003; Pandolfi *et al.*, 2003b; Lotze *et al.*, 2006; Worm *et al.*, 2006). In order to have a better grasp on the extinction rate, research has been focused on the rate of habitat loss, species density and survivorship (Pimm & Raven, 2000). Over-exploitation is thought to be the main cause of the collapse of some fisheries (Worm *et al.*, 2006) but habitat loss, degradation and fragmentation also plays a major role in aquatic species abundance (Nyström *et al.*, 2000; Alongi, 2002; Waycott *et al.*, 2009; Cunha *et al.*, 2013).

Estuaries and marine coastal areas have been focal points of human settlement and use throughout history. Continuous overexploitation, habitat transformation, and pollution have masked the total magnitude of estuarine degradation and biodiversity loss (Jackson *et al.*, 2001a; Pandolfi *et al.*, 2003a; Lotze & Milewski, 2004; Lotze *et al.*, 2005).

Seahorses (Syngnathidae) are emblematic and threatened fish with remarkable morphology and biology, including male pregnancy (Avise *et al.*, 2002). Worldwide, seahorse populations are threatened due to degradation of the habitats, incidental capture by fishing gears (by-catch), and over-exploitation for use in aquarium trade, curiosities and traditional medicines (Vincent, 1996). Seahorses have been reported to be potentially resilient to negative changes, as they have high growth rates, mature at young age and short generation times (Foster & Vincent, 2004) and therefore could recover quickly from population declines (Curtis & Vincent, 2006). However, their distinctive life history, behaviour and ecology might render them vulnerable to such changes, as they provide lengthy and vital parental care for small broods, exhibit low mobility and site fidelity (Foster & Vincent, 2004). In addition, seahorses inhabit shallow, coastal areas worldwide, where anthropogenic disturbances tend to be most frequent and severe (Bell *et al.*, 2003).

Seahorses, as other small marine fish species, contribute to marine biodiversity and ecosystem function, and in order to assess the effects of incidental catch and develop management strategies, it is important to understand their life-history and population parameters (Foster & Vincent, 2004). Understanding seahorse life history becomes particularly important as the entire genus *Hippocampus* has been added to Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), providing trade regulations for these species. Without knowledge of their life-history and population parameters, it will be difficult to develop management strategies to ensure their population persistence in the wild (Foster & Vincent, 2004). The conservation of marine fishes has been discussed since 1996, when the first International Union for Conservation of Nature's (IUCN) Red List assessment was held (Vincent & Hall, 1996). To date, 38 seahorse species assessed by the IUCN Red List, most are described as Data Deficient (26), while others are Vulnerable (10), Least Concerned (1) and Endangered (1) (IUCN, 2014). These classifications demonstrate the gaps of knowledge that there still exists regarding the status of most *Hippocampus* species.

A total of 48 seahorse species have currently been recognized due to morphological, genetic and behavioural taxonomic characters (Lourie, unpublished data). Two sympatric seahorse species inhabit the coastal waters of the North-East Atlantic, including the Mediterranean Sea, the long-snouted seahorse (*Hippocampus guttulatus* Cuvier 1829) and the short-snouted seahorse (*Hippocampus hippocampus* Linnaeus 1758) (Boisseau & Lemenn, 1967; Lourie *et al.*, 1999). These species can be distinguished by morphological traits such as snout size, abdomen, skin colour and adult body size (Curtis, 2006). While *H. guttulatus* has been reported to reach 23cm in total length, *H. hippocampus* maximum reported size is 15cm (Lourie *et al.*, 1999). These species have overlapping areas of distribution but distinct habitat preferences. *H. guttulatus* is generally associated with shallow waters and enclosures (up to 15 meters), and prefers higher habitat complexity, whereas *H. hippocampus* favours deeper areas (up to 55 meters) with lower holdfast availability.

Both species inhabit the Ria Formosa lagoon (Southern Portugal), where the highest population size was recorded for *H. guttulatus*, throughout its range in the early 2000's (Curtis & Vincent, 2005). However, recent field data showed a significant decrease in

seahorse populations within this lagoon (94% and 73% for *H. guttulatus* and *H. hippocampus*, respectively) (Caldwell & Vincent, 2012). Although the causes for such declines remain unknown, human related activities (fisheries, including illegal fishing, anchoring and dredging), natural changes in the dynamics of the Ria (e.g. silting events and shifting currents), as well as broad scale biological process that can impact the recruitment, fecundity and overall abundance of this species, may be the main causes for an overall habitat loss (Curtis *et al.*, 2007).

The Ria Formosa lagoon is a highly productive ecosystem and sustains a great variety of commercial species that have high economic value, as among others, the sparid species (Erzini *et al.*, 2002). South Portugal is a renowned area for tourism and many human related activities occur in the Ria Formosa, such as, aquatic sports and boat traffic including boat anchoring. These activities, combined with fishing (by-catch and illegal fishing) increase environmental disturbance and therefore contributes to the negative pressure on the existing seahorse populations. In fact, the use of fishing gears have a direct (by-catch) and indirect (habitat degradation) impact on both seahorse species. In the Ria Formosa, some natural *Zostera noltii* beds have been replaced by clam farms (Guimarães *et al.*, 2012), harbours, industries and coastal constructions, or dredging to open and maintain navigation channels, thus destroying vast areas of this species habitat (Cunha *et al.*, 2013). These anthropogenic activities and natural events are known to alter the seahorses' habitat conditions and reduce the amount of natural holdfast available, essential for seahorse settlement (2010, Correia pers.obs.).

Although the previous work on seahorse population assessments in the Ria Formosa has highlighted some threats and trends for *H. guttulatus* and *H. hippocampus*, there is a need to understand drivers of seahorse abundance and how they are changing over time. In addition, no long term monitoring has been done in order to determine how seasonal events might influence seahorse density fluctuations.

1.1 Objectives

This thesis aimed to identify activities or events that have negative impacts on seahorse populations through monitoring on a spatial and temporal scale in the Ria Formosa lagoon. In addition, this study aimed to design and test possible new methods and mitigation tools that could help respond to recently reported decline in seahorse numbers and be integrated into a recovery plan.

This thesis was outlined in 8 different chapters structured in a research publication format.

Chapter 1 is a general introduction that provides an overview of the status of relevant seahorse research including recent findings.

Chapter 2 looked at trends in the past decade, adding information to previous surveys (2001-2002 and 2008-2009). The aim of this study was to identify possible causes for seahorse population fluctuations so that research on mitigation actions can be done. This chapter will be submitted to a peer-reviewed journal by the end of the year.

Chapter 3 aimed to test whether a modified UVC technique designed to survey seahorse populations at lower density scenarios was more effective in assessing seahorse abundance at different habitats within the Ria Formosa Lagoon. This chapter has been submitted to a peer-reviewed journal and is awaiting reviewers' comments.

Chapter 4 addressed the spatial and temporal variation in seahorse abundance and aimed to identify the main events that could help explain the recent decline in seahorse populations inside the Ria Formosa. This chapter will be submitted to a peer-reviewed journal by the end of the year.

Chapter 5 focused on evaluating the potential of using photo-identification as a monitoring tool for capture-recapture studies of *H. guttulatus* populations. This chapter was submitted to "*Journal of Fish Biology"* and has been already published (**Correia, M.**, Palma, J., Koldewey, H. & Andrade, J. P. (2014). The use of a non-invasive tool for capture-recapture studies on a seahorse *Hippocampus guttulatus* population. *Journal of Fish Biology* **84**, 872-884.).

Chapter 6 and 7 examined the use of artificial holdfast structures as a habitat recovery tool for seahorse populations. Laboratory work (Chapter 6) and *in situ* experiments (Chapter 7) will help validate and set the basis for the guidelines for habitat enrichment in other areas or even for other seahorse species. Chapter 6 has been submitted to a peer-review journal "Journal of Experimental Marine Biology and Ecology" and was published in October 2013 issue. (**Correia, M.**, Palma, J., Koldewey, H. & Andrade, J. P. (2013). Can artificial holdfast units work as a habitat restoration tool for long-snouted seahorse (*Hippocampus guttulatus* Cuvier)? *Journal of Experimental Marine Biology and Ecology* **448**, 258-264.). Chapter 7 will be submitted to a peer-review journal by the end of the year.

Chapter 2: Long-term monitoring of seahorse populations in the Ria Formosa Lagoon, South Portugal

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2.1 Abstract

Recent findings have reported a significant decrease in abundance of *Hippocampus guttulatus* and *H. hippocampus* in the Ria Formosa lagoon (94% and 73%, respectively). Long term monitoring studies are crucial to track seahorse population trends and identify causes of such fluctuations. During 2010-2013, 16 sites were surveyed and compared with previous data obtained from those same locations in two earlier studies (2001-2002 and 2008-2009). *H. guttulatus* showed a significant population increase in 2010-2013 compared to the previous 2008-2009 surveys and no significant differences in density when compared to 2001-2002. In contrast, no significant differences in *H. hippocampus* densities were found among the 16 sites surveyed throughout the three sampling periods, but this fact might be biased by a high density reported at one site in 2008-2009. Also, an increase in *H. guttulatus* abundance was observed comparing 2010-2013 to 2008-2009 data, which suggest a recovery of this species' population. Of all the tested variables (depth, temperature, holdfast coverage), *H. guttulatus* density only correlated with the percentage of holdfast coverage ($p<0.001$). In addition, when comparing all survey events, the two seahorse species' densities were positively correlated. These results highlights the importance of holdfast availability on seahorse abundance, however other variables could help explain these fluctuations, thus further consideration and investigation into other variables should be done.

Keywords: *Hippocampus guttulatus*; *Hippocampus hippocampus*; long-term survey; habitat changes; population fluctuation

2.2 Introduction

Determining sources of variation in population abundance and identifying the factors causing those fluctuations are crucial questions in ecology (Hunter Jr & Gibbs, 2009). In the past century, much attention has been focused on population biology and the debate about why populations fluctuate (Moran, 1953; May, 1974; Beddington & May, 1977; Kareiva, 1987). This question continues to be contemporary and relevant, particularly due to its implications for the management and conservation of natural resources (Clark, 2010).

Many populations fluctuate due to the natural heterogeneity of the ecosystems (Kolasa *et al.*, 1991). Therefore, studies that characterize the spatial and temporal variability of unexploited populations, and link those to changes in environmental and biological factors can help to understand how various organisms respond to their environment and their relative vulnerability to any new stressors such as direct exploitation or habitat degradation (Martin-Smith & Vincent, 2005). Baillie *et al.* (2004) reported that most marine extinctions were attributed to exploitation (55%) and habitat loss/degradation (37%). Nevertheless, the effects of habitat loss are difficult to separate from other impacts as they are commonly only one of multiple stressors. For example, human-induced habitat loss has been linked to coastal development with associated pollution, leading to sediment loading and added nutrients inputs (Short & Wyllie-Echeverria, 1996).

Estuaries and marine coastal areas, such as the Ria Formosa lagoon, South Portugal, are naturally variable ecosystems that are also focal points of human settlement and marine resource use throughout history, exposing them to additional, anthropogenic impacts. Continuous overexploitation, habitat transformation, and pollution have masked the overall magnitude of habitat degradation and biodiversity loss throughout the world's estuaries (Jackson *et al.*, 2001; Pandolfi *et al.*, 2003; Lotze & Milewski, 2004; Lotze *et al.*, 2005). The Ria Formosa lagoon is a highly productive estuarine ecosystem, sustaining a wide variety of commercial species with high economic value (e.g. sparid species such as *Sparus aurata*, *Diplodus sargus*, *Diplodus vulgaris*, Moronidae species (e.g. *Dicentrarchus labrax*) and several different flat fish) (Ribeiro *et al.*, 2006). However, the use of fishing gears targeting such commercially important species have direct (by-catch) and indirect (habitat degradation) impacts on non-targeted species as well. Southern Portugal is also a renowned

tourist area with many associated habitat impacting activities including aquatic sports, boat traffic and boat anchoring. In addition, habitats within the lagoon have been further transformed and degraded through other activities. Many natural *Zostera noltii* beds have been replaced by clam farms (Guimarães *et al.*, 2012), and other seagrass beds have been destroyed by coastal construction and dredging to open and maintain navigation channels (Cunha *et al.*, 2013).

Typically, seahorses have a sparse distribution, low mobility, small home ranges, low fecundity, lengthy parental care and mate fidelity, rendering them vulnerable to overfishing and habitat damage (Foster & Vincent, 2004). Thus, compared to other fish species, seahorses have a life history that make them particularly vulnerable to the combined pressure that may result from natural shifting habitats and anthropogenic activity which occur in the estuaries and coastal environments they inhabit. Most seahorse species use their prehensile tail as a means to grasp different holdfasts, from sponges to coral, seagrass, mangrove branches and even artificial structures thus relying on some degree of habitat structure (Foster & Vincent, 2004; Harasti *et al.*, 2010; Hellyer *et al.*, 2011). Although these fishes are described as sedentary, they can move at least 150m from its original area (Caldwell & Vincent, 2013) and have a home range up to 400m^2 (Garrick-Maidment *et al.*, 2011). These movements might be influenced by habitat specificity and presence of other seahorses. In fact, in specific months, these seahorses might adjust their location depending on holdfast and food availability, density, fishing activities and weather conditions (Correia *et al.*, 2014).

Two sympatric seahorse species inhabit the Ria Formosa lagoon: the long-snout seahorse (*Hippocampus guttulatus*) and the short-snout seahorse (*H. hippocampus*). These seahorse populations have been monitored in the Ria Formosa since the early 2000's (Curtis & Vincent, 2005; Caldwell & Vincent, 2012; Correia *et al.*, 2014). The biggest population size recorded for this species, throughout its range, was recorded by Curtis & Vincent (2005). However, recent field data published by Caldwell & Vincent (2012) showed a significant population decrease of 94% for *H. guttulatus* and 73% for *H. hippocampus* in this lagoon. Initially, the causes for such declines remained fairly unknown however, more recent observations pointed human related activities (fisheries, including illegal fishing, anchoring

and dredging) and natural changes in the Ria's dynamics (e.g. silting events and shifting currents) as the main causes for an overall habitat loss (Curtis *et al.*, 2007).

Although the previous work in seahorse population census has brought important findings regarding trends and threats that *H. guttulatus* and *H. hippocampus* face, research is lacking to help identify other events that could lead to the decline in seahorse abundance. As long term monitoring studies are crucial to confirm seahorse population trends and identify possible causes for such fluctuations, this study aimed to determine if seahorse populations in the Ria Formosa lagoon have continued to decline since the previous census, and at the same time to clarify if the measured environmental variables are associated with population changes over time.

2.3 Material and Methods

2.3.1 Underwater Visual Census surveys

In the years of 2010, 2012 and 2013 Underwater Visual Census (UVC) surveys were conducted at 16 sites (Figure 1) that had been previously surveyed in the Ria Formosa Lagoon, south Portugal (36° 59' N; 7° 51' W). The UVC method used was the same as described by Curtis & Vincent (2005) and Caldwell & Vincent (2012). Surveys were performed in high slack tides, when there is less turbidity and weaker currents. A GPS unit was used to determine the locations of each study area and the same bearing was taken while laying each transect so that the same area could be consistently covered on each sampling occasion. Whenever a seahorse was found, its species was recorded along with three environmental variables that were recorded in previous surveys by Curtis & Vincent (2005) and Caldwell & Vincent (2012): water depth, water temperature (at depth), and the percentage cover of holdfasts (i.e. the percentage of the benthos within a $1m^2$ quadrat centered around the seahorse that was covered by either living or non-living material that could be grasped by a seahorse's tail).

Figure 2.1: Location of the 16 sites surveyed in 2001-2002, 2008-2009 and 2010-2013.

2.3.2 Changes in species density among survey periods

To assess whether seahorse densities had changed over time within the Ria Formosa lagoon, species densities from the latest UVC surveys (2010, 2012, and 2013) were compared with surveys at the same sites from 2001-2002 (Curtis & Vincent 2005) and 2008-2009 (Caldwell & Vincent 2012). Seahorse densities (number of individuals per m^2), rather than abundances, were compared to account for differences in surveyed areas per site in each time period, as in Caldwell & Vincent (2012). One density was calculated for each species at each site by dividing the abundances of that species at that site by the total area surveyed in the site. These densities were then compared for all 16 sites during the three time periods. Since species densities were non-normal and transformations for normality were unsuccessful, densities were compared among the three time periods using a non-parametric Friedman test to account for repeated measures at each of the 16 sites. When Friedman tests were significant, Wilcoxon signed rank tests were used to identify which of the three time periods differed, using a Bonferroni corrected alpha value of 0.0167 to account for multiple comparisons (i.e. 0.05/3). R statistical software was used for both the Friedman test and Wilcoxon post-hoc tests (‘friedman.test’ and ‘Wilcox.test’ functions in the ‘stats’ R package; R Core Team, 2013).

2.3.3 Environmental correlates of species density changes

To identify whether changes in seahorse densities were associated with any changes in their environment, the differences in the two species densities between successive surveys were compared with differences in the three environmental variables collected during those same survey periods (i.e. depth, temperature, and percent holdfast cover). The differences in seahorse densities and environmental conditions were calculated between the latest survey period and the middle survey period (2010-2013 vs. 2008-2009) and between the middle survey period and the earliest survey period (2008-2009 vs. 2001-2002), resulting in two sets of differences (i.e. changes in seahorse density and environment) for each of the 16 sites in common.

Changes in density were compared with changes in environment using generalized linear mixed effects models (GLMM), fit to the data using a forward stepwise process informed by Spearman rank correlations. Before fitting GLMM's, non-parametric Spearman rank correlations were used to compare changes in individual environmental correlates with changes in seahorse densities and to identify any co-linearity among the environmental variables (using 'cor.test' function in the R 'stats' package; R Core Team, 2013). A non-parametric test was used as the data were non-normal and transformations for normality were unsuccessful. Results of the Spearman rank correlations were then used to inform model selection. Starting with the variable with the highest significance (i.e. lowest P-value), GLMMs were created in a forward stepwise process, adding variables with successively lower significance. GLMMs were used instead of generalized linear models (GLMs) to include the effect of site as a random factor. Each GLMM used a Gaussian distribution with a log-link function within the 'lmer' function of the R package 'lme4' (Bates *et al.*, 2014). Successive GLMM's were compared with previous models using the 'ANOVA' function of the 'stats' R package (Team, 2013) to test for differences in the amount of deviance explained (Zuur *et al.*, 2009). Variables and/or their interactions were only retained if their addition explained significantly more deviance according to a χ^2 test.

2.4 Results

The most recent surveys of seahorse densities in the Ria Formosa lagoon (2010-2013) revealed significant increases in *H. guttulatus* densities since the previous 2008-2009 surveys, but no significant changes in *H. hippocampus* densities among the 16 sites surveyed (Figure 2). According to a Friedman test, there were significant differences in *H. guttulatus* densities among the three survey periods ($p < 0.001$). Wilcoxon signed rank post-hoc tests further revealed that the differences in *H. guttulatus* densities were due to a significant decrease between the earliest survey and the middle survey (2001/2002 vs. 2008/2009; $p < 0.001$), followed by a significant increase between the middle survey and the most recent survey (2008/2009 vs. 2010-2013; $p = 0.002$), with no difference between the earliest survey and the most recent survey after applying a Bonferonni correction (2001/2002 vs. 2010-2013; $p = 0.025$). Although a Friedman test also indicated there were significant differences in *H. hippocampus* densities ($p = 0.034$), none of the Wilcoxon signed rank post-hoc tests were significant after applying the Bonferroni correction (all $p > 0.028$), suggesting there were no significant changes in *H. hippocampus* densities between each of the three survey periods.

Both *H. guttulatus* and *H. hippocampus* densities fluctuated from the higher mean value observed in 2001-2002, decreasing in 2008/2009 and increasing from that point to 2010-2013 (Table I). Although *H. guttulatus* densities increased from 2008/2009 to 2010-2013, the mean value still falls below the ones reported in 2001-2002. In contrast, *H. hippocampus* mean densities were higher in 2010-2013.

Table 2.I: Densities (mean and maximum) and % occupancy of two seahorse species in 16 sites surveyed in three time periods in the Ria Formosa lagoon.

	H. guttulatus			*H. hippocampus*		
	2001-2002	2008-2009	2010-2013	2001-2002	2008-2009	2010-2013
Mean density (/m^2)	0.090	0.007	0.053	0.007	0.002	0.008
Occupancy (%)	93.75	37.5	75.0	37.5	31.25	68.75
Maximum density (/m^2)	0.339	0.035	0.183	0.033	0.019	0.024

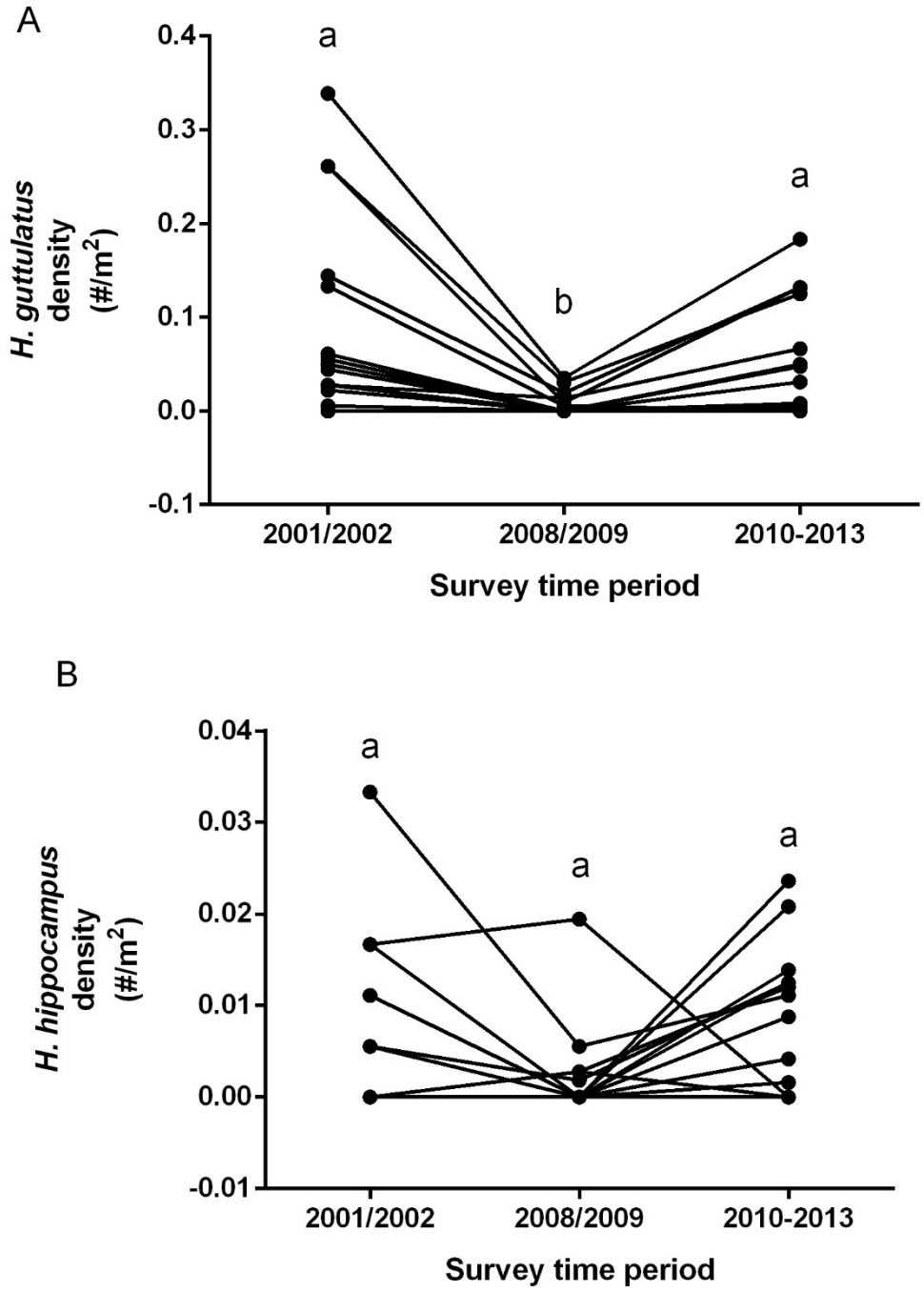

Figure 2.2: Comparisons of *H. guttulatus* (A) and *H. hippocampus* (B) densities found during three underwater visual census survey periods of 16 sites within the Ria Formosa lagoon, Portugal. Each circle represents one of the 16 sites and surveys with the same letter are not significantly different (i.e. Wilcoxon signed rank post hoc test > 0.0167).

The only change in the environment of the 16 surveyed sites between successive survey periods that was correlated with changes in either of the two seahorse species densities was between percentage cover of holdfasts and *H. guttulatus* densities (Figure 3). Spearman rank correlations among all five variables (i.e. changes in the three environmental variables and the two seahorse species densities) were only significant for two relationships: 1) changes in *H. guttulatus* density were positively correlated with changes in holdfast coverage ($p < 0.001$) and 2) densities of the two seahorse species were positively correlated ($p = 0.002$). According to χ^2 tests comparing GLMM's with and without the addition of further environmental variables, none of the more complex models explained more of the deviance in *H. guttulatus* density changes than the model with percentage holdfast cover alone (all $p > 0.160$). None of the environmental variables measured in the three surveys explained a significant amount of the deviance in *H. hippocampus* density changes compared to the null GLMM including only the random effect of site.

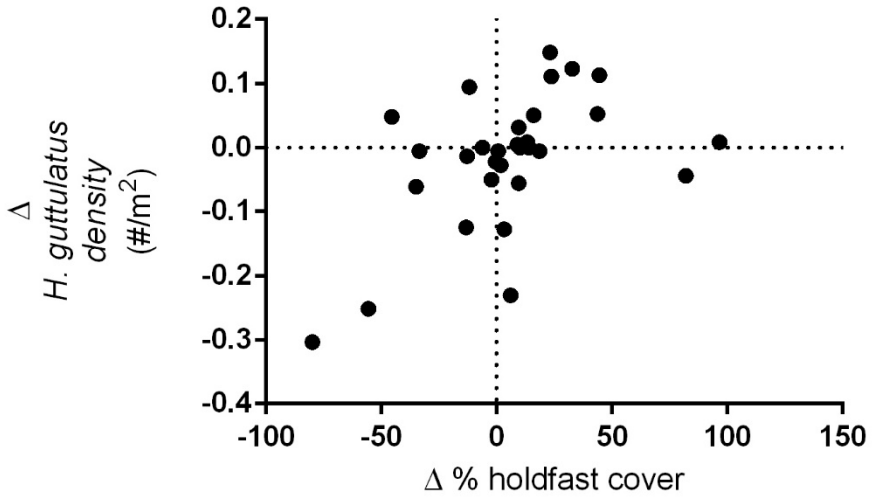

Figure 2.3: Changes in *H. guttulatus* densities associated with changes in the percentage cover of holdfasts between three successive underwater visual census survey periods at 16 sites within the Ria Formosa lagoon, Portugal.

2.5 Discussion

The latest seahorse population survey in the Ria Formosa identified a fluctuation in *H. guttulatus* and *H. hippocampus* abundance in a decade. Although a dramatic decrease in abundance has been reported in previous studies for *H. guttulatus* (94%) and *H. hippocampus* (73%) (Caldwell & Vincent, 2012), the seahorse population abundance for both species appears to be increasing. The mean *H. hippocampus* density observed in this study was higher than those reported in 2001-2002. The *H. guttulatus* population seems to be increasing but still lower than the abundances recorded in 2001-2002. Temporal fluctuations have been recorded for several fish species but most studies focused on those of commercial importance (Beare *et al.*, 2004; Daufresne & Renault, 2006; Costa *et al.*, 2012; Mariani *et al.*, 2013). The causes of such fluctuations are generally hard to determine as many variables have to been taken in consideration. Among those variables, exploitation, habitat loss/degradation and climate change have been reported as the main causes for these fluctuations and can ultimately lead to extinction (Dulvy *et al.*, 2003). In fact, several authors characterized the minimum amount of habitat required for a population of a particular species to persist as the "extinction threshold". This threshold assumes that, in a degraded habitat, a small additional loss of habitat leads to fragmentation and isolation, which can negatively influence the reproduction and recruitment of the species (With & King, 1999; Fahrig, 2002).

Several studies have identified fluctuations in the abundance of other seahorse species such as *H. abdominalis* (Martin-Smith & Vincent, 2005), *H. reidi* (Freret-Meurer & Andreata, 2008) and *H. zosterae* (Masonjones *et al.*, 2010). Nevertheless no variable, either human influenced or natural, has clearly been identified as the cause for those fluctuations. However, several causes have been suggested, such as human related activities, that can have a direct (costal constructions, exploitation) or indirect impact (changes in water quality) in seahorse populations (Masonjones *et al.*, 2010); natural fluctuations due to recruitment failure (Martin-Smith & Vincent, 2005); and changes in holdfast availability (Rosa *et al.*, 2007)

In the Ria Formosa, human related activities (e.g. fisheries including illegal fishing, anchoring and dredging) and natural changes in the lagoon's dynamics (e.g. silting events and shifting currents), seem to be the main causes for an overall habitat loss (Curtis *et al.*, 2007). The Ria Formosa sustains a relevant clam farming activity (Guimarães *et al.*, 2012),

harbours, shipyards, coastal constructions and episodic dredging activity (to open and maintain navigation channels), which combined are responsible for the destruction of vast areas of potential seahorse habitat (Cunha *et al.*, 2013). The combination of these anthropogenic activities and natural events are known to alter the seahorses' habitat conditions and reduce the available amount of natural holdfasts, essential for seahorse settlement (2010, Correia pers.obs.). In the past, both seahorse species were sometimes sold as curiosities (Project Seahorse, 2003a; b), but this market is now prohibited in Portugal. Nevertheless, there has been anecdotal reports of an illegal trade of dried specimens, although the significance of this on seahorse populations is unknown. In addition, local authorities have recently reported an increase in bottom trawling and beach seine fisheries. Although these activities are forbidden in the Ria Formosa (Erzini *et al.*, 2002), these are responsible for seahorse by-catch (Curtis *et al.*, 2007), however with minimal expected impact in overall seahorse mortality. Gill and trammel nets are however allowed to use in the lagoon. The use of these nets have a direct impact on the local seahorse populations, as the seahorses get tangled in the net, when hauled on board they are not returned (Correia, pers. obs.).

Habitat changes have been reported in the Ria Formosa, where seagrass beds have been declining in the past 20 years, with 75% reduction in the distribution of *Zostera noltii* (Cunha *et al.*, 2014). Moreover, the distribution of seagrass beds has been reported to migrate in response to natural and human-induced activities (Cunha *et al.*, 2005). Considering that seahorses have been reported to favor seagrass as preferred habitat (Curtis & Vincent, 2005), this decrease in seagrass abundance should have a negative impact on seahorse populations, particularly for *H. guttulatus* (Ribeiro *et al.*, 2006). In face of seagrass depletion, seahorses might occupy other habitats as an alternative, causing the dispersal of seahorse populations. This might add some difficulty when surveying these areas in long term, so a wider area should be surveyed in order to minimize the effects of these inter-annual habitat changes (Chapter 3). *H. guttulatus* has been reported to be unspecific regarding holdfast preference and have been observed to grasp a variety of holdfast, both natural and artificial (Curtis & Vincent, 2005). Moreover, during the surveys, the most abundant bottom coverage observed varied from shells, to tunicates, tube dwelling polychaetes (*Sabella* sp.) and artificial holdfasts. These holdfasts were patchy distributed where higher densities of *H. guttulatus* were reported (Correia, pers. obs), a fact that highlights the importance of surveying the same

area for long term monitoring purposes (Chapter 3). Also, the most recent surveys of seahorse densities in the Ria Formosa lagoon revealed significant increases in *H. guttulatus* densities, but no significant changes in *H. hippocampus* densities among the 16 sites surveyed. This result might be biased by the high density observed at one site in 2001-2002. If this site were to be removed from the statistical analysis, the outcome would be the significant differences in densities between 2001-2002 and the two other survey events (2008-2009 and 2010-2013). This might be due to the relatively low abundance of *H. hippocampus* which made it difficult to provide enough data to reveal any strong statistical significance. This could be minimized by expanding the survey area and eliminating the randomness behind the UVC methodology used in this study, to increase the probability of finding more seahorses (Chapter 3).

H. guttulatus densities correlated positively with holdfast availability, the only variable that showed statistical significance as a predictor for this species' abundance, a fact that agrees with Curtis & Vincent (2005). This detail stresses the importance of holdfast availability for seahorse settlement. Considering that the Ria Formosa lagoon is a highly hydrodynamic system, where average maximum current speed can go up to 1.25 $m \cdot s^{-1}$ (Pacheco *et al.*, 2010), and that seahorses have low mobility, they need holdfasts to be widely available to prevent them to be dragged away from their preferred habitats (Curtis & Vincent, 2005). Artificial seagrass has been used in many countries as a method to replace the damaged natural seagrass ecosystem providing marine habitat for various marine organisms, nursery ground for juveniles, and habitat and protection for small fishes (Sogard, 1989; Sogard & Able, 1994; Kenyon *et al.*, 1999; Lee *et al.*, 2001; Shahbudin *et al.*, 2011). Lately, artificial holdfasts were tested as habitat restauration tool for *H. guttulatus*, firstly in captivity (Chapter 6) and then *in situ* (Chapter 7), with promising results. Although these structures have proven their usefulness to promote seahorse settlement, they should be mainly considered for heavily degraded habitats and as temporary mitigation (Chapter 7).

In contrast, *H hippocampus* did not correlate with percentage of bottom coverage. This fact agrees with Curtis & Vincent (2005) that reported a preference for lower holdfast availability areas. The reported differences in habitat preferences between *H. guttulatus* and *H. hippocampus* might be explained by the interspecific foraging behaviour. *H. hippocampus* is more active as swimmer and predator of planktonic and epibenthic prey, while *H. guttulatus*

is more sedentary and uses ambush as a predation strategy (Curtis & Vincent, 2005). This behaviour was also observed in this study.

The densities of the two species were positively correlated, which suggests that both species have the same response to the stressor. Since *H. hippocampus* densities were not correlated to holdfast availability, it seems that other variables might be responsible for these fluctuations. According to Shelton & Mangel (2011), species interactions, such as predator–prey interactions, can be the cause of population fluctuation. Although there are only two documented predators of *H. guttulatus* and *H. hippocampus,* i.e. the common octopus (*Octopus vulgaris*) and the loggerhead sea turtle (*Caretta caretta*) (Kleiber *et al.,* 2011), cuttlefish (*Sepia officianalis)* have observed feeding on these seahorse species (Correia, pers. obs). Octopus and cuttlefish were commonly seen in 2008-2009 and 2010-2013 surveys, but these predators tend to be opportunistic feeders (Kleiber *et al.,* 2011). A decrease in prey abundance might drive this species to feed on seahorses, but considering the nature of the Ria Formosa, i.e., nursing ground for several species, this seems unlikely to be the cause.

In this study, all seahorse population surveys were done from July to November, thus no information is available on seahorse abundance on the remaining months. Therefore, in order to overcome the existing gaps in the seahorse population surveys, spatial and temporal distribution of the wild populations of the long-snout seahorse (*H. guttulatus*) and the short-snout seahorse (*H. hippocampus*) should be assessed throughout one year period, to identify the environmental variables that influences the seahorse population dynamics (Chapter 4).

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Chapter 3: Assessing seahorse density: A comparison between two underwater visual census techniques

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3.1 Abstract

Underwater visual census (UVC) have been used to document a wide variety of marine fish species. Two different UVCs (UVC1 developed previously and UVC2 developed for this study) were conducted at three sites in the Ria Formosa lagoon (Portugal) to compare densities of the seahorses *Hippocampus guttulatus* and *H. hippocampus* and holdfast availability. The density of *H. guttulatus* was significantly ($P<0.05$) higher using UVC2 compared to UVC1 at two of the three sites studied. There were no significant ($P>0.05$) differences in estimated holdfast availability using both UVC methods. The UVC method developed here will be more appropriate for long-term monitoring of seahorses, particularly for low density populations.

Keywords: *Hippocampus guttulatus; Hippocampus hippocampus*; underwater survey; population densities.

3.2 Introduction

The first underwater visual census (UVC) methods were performed by Brock (1954) and set the basis for most studies on fish ecology. UVCs have since been used to document a wide variety of marine fish species, especially those exploited in fisheries (Russ, 1984; Kulbicki, 1998; Ferreira *et al.*, 2004; Mayo & Jackson, 2006; McClanahan *et al.*, 2009). The non-destructive nature of this technique makes it appealing to fish monitoring as it is fisheries-independent, quick and relatively inexpensive (Watson & Quinn, 1997). UVCs have also

been reported to be an appropriate technique for estimating the density of shallow-water reef fish and is used on a global scale (Floeter *et al.*, 2007; Evans *et al.*, 2008; Leopold *et al.*, 2009; Andrefouet & Wantiez, 2010), including in the Mediterranean (García-Charton *et al.*, 2004; Stobart *et al.*, 2007). This method involves systematic data collection in the surveyed area, enumerating the animals and collecting environmental information (Curtis *et al.*, 2004) and it can be used for the estimation of relative densities, biomass, and distribution of fish populations (Andaloro *et al.*, 2011; Cenci *et al.*, 2011; Pelletier *et al.*, 2011).

However, underwater visual surveys present specific challenges, including restricted surveying time spent underwater, observer mobility and underwater visibility (Charton *et al.*, 2000; Williams *et al.*, 2006). Thus, UVC method has been adapted to monitor fish populations depending on the target fishes' biology, behaviour and surveyed area (Mapstone & Ayling, 1998; Samoilys & Carlos, 2000; Ordines *et al.*, 2005).

UVC has been widely used for surveys of particular fish families and species (De Raedemaecker *et al.*, 2010; Kulbicki *et al.*, 2010), including seahorses (Bell *et al.*, 2003; Curtis *et al.*, 2004; Curtis & Vincent, 2005; Freret-Meurer & Andreata, 2008; Caldwell & Vincent, 2012). In the early 2000's, Curtis & Vincent (2005) used UVC to describe the highest seahorse densities ever recorded in the Ria Formosa lagoon and elsewhere. The protocol used in that study was designed to suit this high seahorse density and the wide distribution of the focal species throughout the lagoon. However, a more recent study of the same locations within the Ria Formosa, using the same UVC method as Curtis and Vincent (2005), showed a significant decrease in seahorse density: 94% and 57% for *Hippocampus guttulatus* Cuvier 1829 and *Hippocampus hippocampus* Linnaeus 1758, respectively (Caldwell & Vincent, 2012). Most seahorse species are found at low densities and patchily distributed (Perante *et al.*, 2002; Bell *et al.*, 2003; Martin-Smith & Vincent, 2005; Freret-Meurer & Andreata, 2008; Harasti *et al.*, 2012) meaning the UVC method described by Curtis & Vincent (2005) may be more challenging and/or less effective as it can underestimate the actual seahorse density. Therefore, this study aimed to test whether a modified UVC technique designed to effectively survey seahorse populations at lower density scenarios was more effective in assessing seahorse density at different habitats within the Ria Formosa Lagoon.

3.3 Material and Methods

Two seahorse species occur in the Ria Formosa lagoon (South Portugal), the long-snouted seahorse (*H. guttulatus*) and the short-snouted seahorse (*H. hippocampus*). These species are easily distinguishable by their size and morphological traits including coronet shape, number of trunk rings (Lourie *et al.*, 2004) and skin colour patterns. These are two species that share the same distribution in shallow, coastal waters of the north-eastern Atlantic Ocean and Mediterranean Sea (Lourie *et al.*, 2004). However, they have distinct habitat preferences: while *H. guttulatus* has been reported to prefer higher habitat complexity such as seagrass meadows, *H. hippocampus* was mostly found in low complexity sandy habitats (Curtis & Vincent, 2005).

Underwater surveys were were conducted at three locations in the Ria Formosa lagoon, south Portugal (36°59'N, 7°51'W) on three occasions (Figure 1).

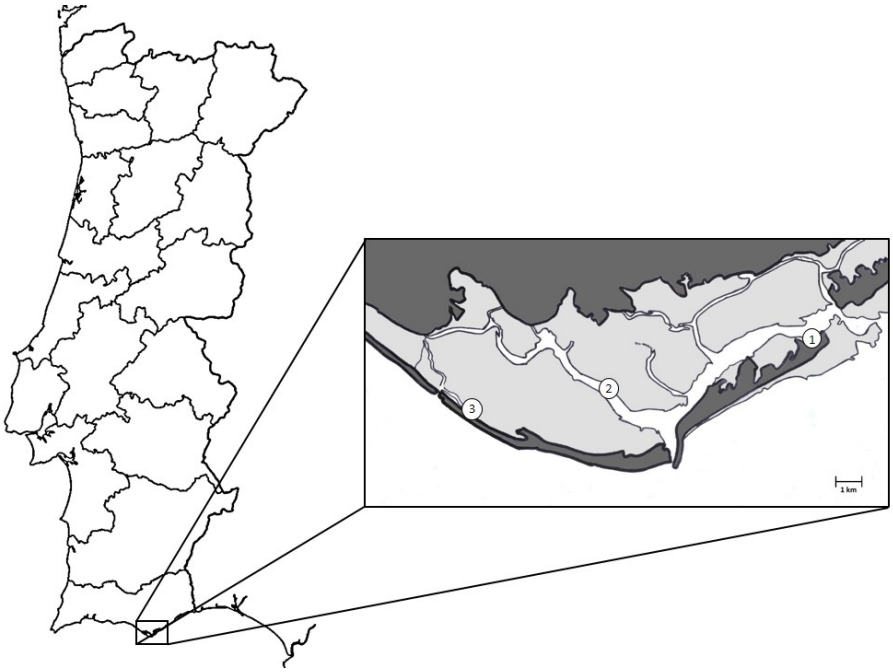

Figure 3.1: Sites location in the Ria Formosa lagoon. Site 1 (1), Site 2 (2) and Site 3 (3).

The surveyed sites in this study were chosen due to their differences in habitat characteristics (Table I). A GPS unit was used to accurately determine the location of each study site and during the site delineation. The same compass bearing was taken while laying each transect so that the same area could be consistently covered on each sampling occasion.

Table 3.I: Description of each surveyed site including; depth (m), water flow ($m.s^{-1}$), substrate, habitat complexity, holdfast distribution and nominal human impact.

Location	Depth (m)	Hydrodynamics ($m.s^{-1}$)	Substrate	Habitat complexity	Holdfast distribution	Human impact
Site 1	5 - 6	High (0.5 - 1)	Sand	High	patchy	High (fisheries and boat anchoring)
Site 2	3 - 4	Medium (0.3 - 0.7)	Mud	Medium	patchy	Medium (occasional fisheries)
Site 3	2 - 3	Low (0.1 - 0.5)	Sand	Low	Homogeneous	Low (no human activity)

On each sampling occasion, the two survey techniques were performed simultaneously in order to minimize bias derived from individual seahorse's daily movement. The UVC method used by Caldwell & Vincent (2012)), hereafter described as UVC1, was conducted first at each site. In this method three 30 m by 2 m belt transects randomly placed >5m apart were surveyed by two divers (Figure 2), with a total area of 180 m^2 per site. The second UVC method, hereafter described as UVC2, consisted of two 30 m transect belts, placed in parallel, 4 meters apart. Each diver surveyed each transect belt, firstly covering the right side and then the left side. In this process, each diver covered an area of 120 m^2, with a total combined area of 240 m^2 at each site. The same divers performed the two UVC methods in order to minimize observational bias. Whenever a seahorse was found, information on species, sex, size, substrate type, holdfast type and overall seabed habitat (in a $1m^2$ diameter area at each seahorse location) was recorded. Seahorse density was calculated for each UVC transect at each site and a Student t-test was used to test for significant differences between UVC methods and sites (Zar, 1999).

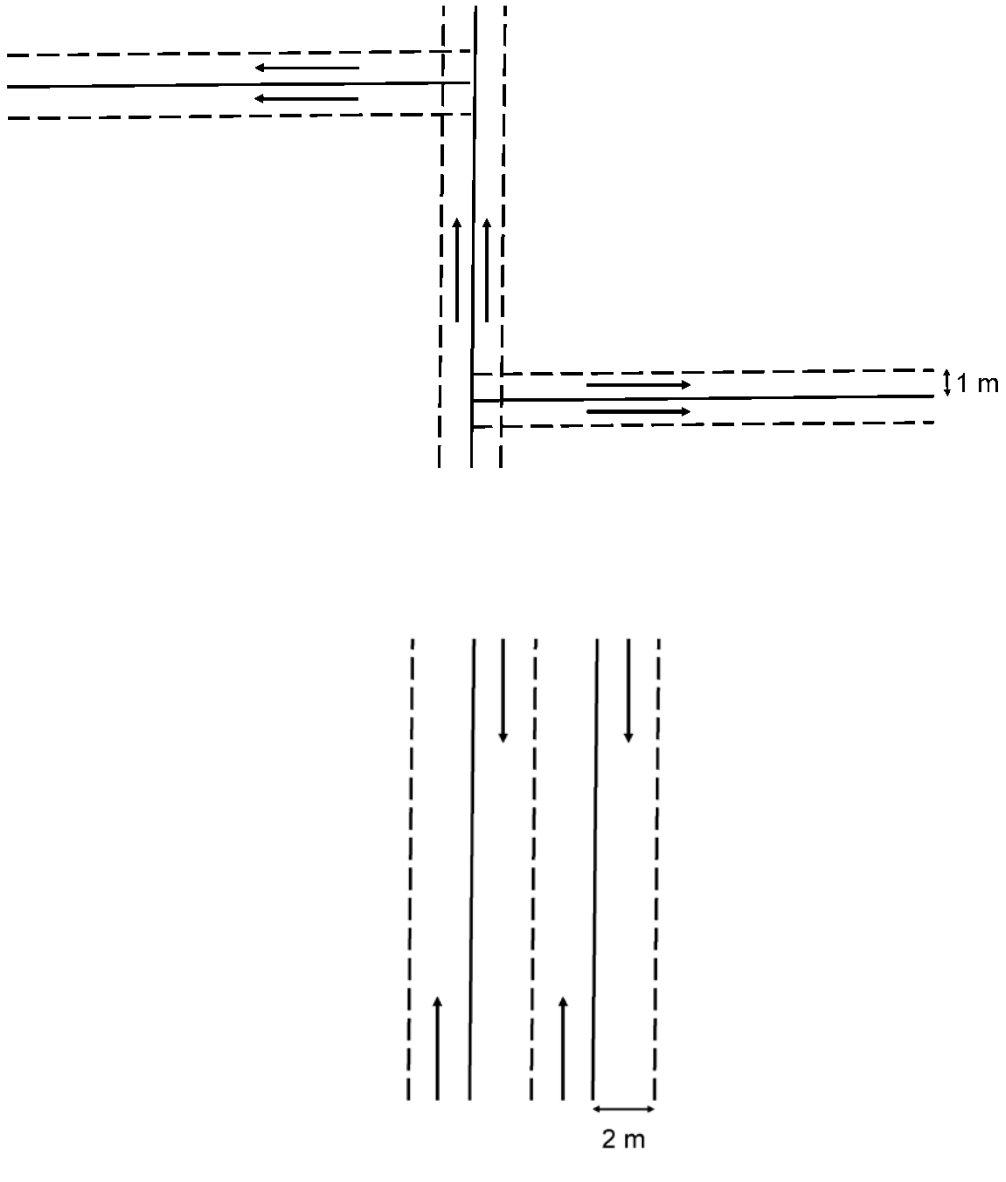

Figure 3.2: Design of the two UVC methods tested. a) Previously used methodology (UVC1) and b) new proposed methodology (UVC2). Arrows indicate diver's direction during survey.

3.4 Results

The observed overall mean ± S.D. for seahorse densities (both species pooled together) using UVC1 was 12 ± 4, 3 ± 1 and 11 ± 1 seahorses per transect, whereas, using UVC2 was 23 ± 1, 21± 8 and 14 ± 5 seahorses, at Site 1, 2 and 3, respectively. *H. guttulatus* and *H.*

hippocampus densities at each site, using the two different UVCs are presented in Figure 3. The observed *H. guttulatus* densities were significantly different between methods at Site 1 ($t_{(4)}$=4.492, P<0.05) and Site 2 ($t_{(4)}$=4.053, P<0.05), but there was no significant difference between methods at Site 3 (P>0.05). There were no significant differences (P>0.05) between methods at the three sites for *H. hippocampus*.

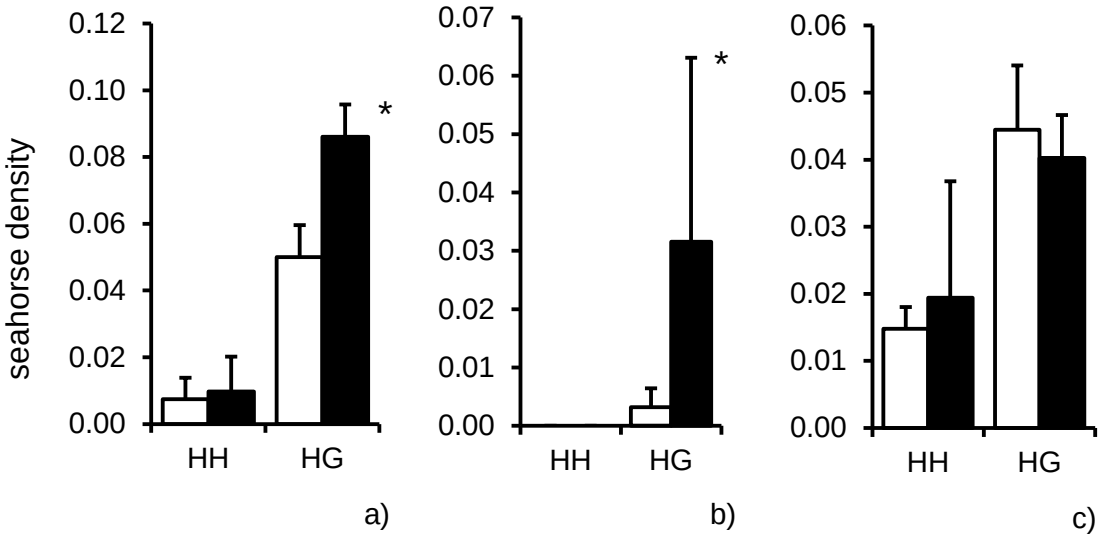

Figure 3.3: Seahorse density (seahorse m^{-2}) of the two seahorse species, *H. hippocampus* (HH) and *H. guttulatus* (HG), in Site 1 (a), Site 2 (b) and Site 3 (c), using two different UVCs (□ – UVC1 and ■ – UVC2) re-sighted. Statistical differences (P<0.05) are presented by (*). Bars represent standard deviation.

The frequencies of each type of seahorse holdfast at each site are shown in Figure 4. The most common holdfast at Site 1 was shells, whilst the most common holdfast at Site 2 and Site 3 were ascidians. There were no significant differences (P>0.05) between UVC methods for any of the holdfasts observed.

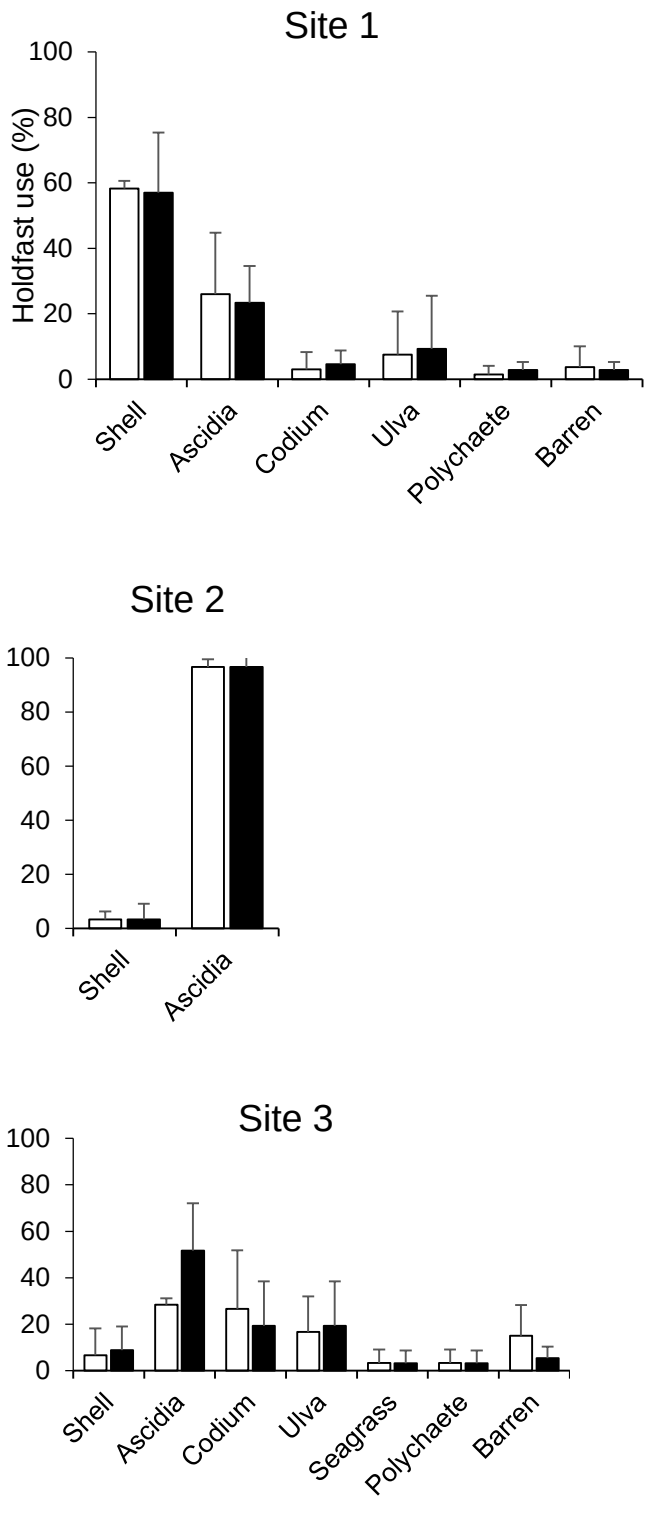

Figure 3.4: Seahorse holdfast use (%) at each site, using UVC1 (□) and UVC2 (■). Statistical differences ($P<0.05$) are presented by (*). Bars represent standard deviation.

3.5 Discussion

Considering the low mobility, small home range and patchy distribution reported for seahorse species, the UVC method used to document their density needs to be appropriate to the species and characteristics of the site, as reported for several species (De Girolamo & Mazzoldi, 2001; Harvey *et al.*, 2004; Colton & Swearer, 2010). Our results showed higher seahorse density when using the UVC2 method developed in this study, but only for one of the two seahorse species found in the study site, i.e., *H. guttulatus*. This may have been due to the patchy distribution reported for this species (Curtis & Vincent, 2006) related to habitat constraints such as low holdfast availability. As seahorses rely on their prehensile tail to grasp on holdfasts to enhance their cryptic behaviour and cope with high hydrodynamic environments (Curtis & Vincent, 2006), this behaviour is also responsible for their low mobility and small home ranges (Curtis & Vincent, 2006; Caldwell & Vincent, 2013). Therefore, ensuring that the areas with available holdfasts are also represented in the survey is of utmost importance, as they usually sustain higher probabilities for seahorse occurrence. Failing to survey these areas, might skew and bias any calculated seahorse densities thus misleading the actual status of the targeted populations.

The different habitat preferences of the seahorse species that occur in the Ria Formosa lagoon should be considered as part of the UVC method used. Although both species preferred using covered microhabitats as holdfasts, *H. hippocampus* was observed more often on bare surfaces than *H. guttulatus* (Curtis & Vincent, 2005). This could explain why there were no statistical differences in density for this species when using the two different UVC methods. In contrast, *H. guttulatus* has been reported to be associated with higher habitat complexities and to be holdfast generalist. Therefore, UVC2 seems to be more suitable for surveying *H. guttulatus* populations as it focuses on target areas that correspond to this species' occurrence.

Within the Ria Formosa lagoon, suitable holdfast areas are patchily distributed, confined to small areas and usually located close to the main channels. These areas create an environment that seems to be suitable for seahorse settlement and therefore should be included in the sampling areas, when performing a population census using UVC techniques. The combined effect of seahorse patchiness and the randomness of the transect positioning and direction of UVC1, used by Curtis & Vincent (2005) and Caldwell & Vincent (2012), may underestimate

the number of seahorses occurring in these important areas, providing potentially biased density information. Nevertheless, this method is suitable for surveying areas with high holdfast availability, holdfasts evenly distributed, and in sites with high seahorse density.

Considering the observed fluctuations in seahorse populations within the Ria Formosa lagoon in the last 10 years (Curtis & Vincent, 2005; Caldwell & Vincent, 2012), we conclude that the UVC2 method is more appropriate for a long-term monitoring programme at this site as it was shown to cope better with low density population scenarios. This method is also likely to have wider applicability for surveying seahorses that tend to be found at low densities globally (Perante *et al.*, 2002; Bell *et al.*, 2003; Martin-Smith & Vincent, 2005; Freret-Meurer & Andreata, 2008; Harasti *et al.*, 2012).

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Chapter 4: Spatial and temporal variation on the abundance of two sympatric seahorse species in the Ria Formosa lagoon, South Portugal

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4.1 Abstract

Recent findings have reported a significant decrease in abundance of two sympatric species (*Hippocampus guttulatus* and *Hippocampus hippocampus*) in the Ria Formosa and no direct causes were clearly identified. This study aimed to identify fluctuations in the local seahorse populations through monthly surveys over a course of a year, in order to identify some of the drivers behind the seasonal fluctuations. Six sites were chosen based on their different habitat characteristics, ranging from barren areas to high algal coverage. The highest *H. guttulatus* abundances were recorded at sites with higher holdfast availability and depth ranging from 3 to 6 meters while *H. hippocampus* were observed at highest numbers at sites with less holdfast availability and patchy distributed. In most sites, seahorse density decreased during the summer months, i.e. from May to August and increased from that point onwards. Holdfast use changed in the sites surveyed, according to the respective habitat characteristics. This study identified environmental variables that influenced the abundance of seahorse population, i.e., holdfast availability, depth and temperature, in the Ria Formosa lagoon, underlining the importance of monitoring populations over a course of a year in order to avoid bias due to seasonal fluctuations.

Keywords: *Hippocampus guttulatus*; *Hippocampus hippocampus*; long term survey; seasonal changes; population abundance

4.2 Introduction

Seahorses have been reported as a flagship species for lagoon and estuarine conservation (Martin-Smith & Vincent, 2005), areas that are largely affected by many events including overfishing (either as targeted species or by-catch), degradation and habitat lost (Vincent, 1996; Baum *et al.*, 2003; Bell *et al.*, 2003; Martin-Smith & Vincent, 2006). Seahorses are used in traditional medicine, as curios and as ornamental fish for the aquarium trade (Vincent, 1996; Koldewey & Martin-Smith, 2010). All *Hippocampus* species were included in the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species in 2004. Of the 38 seahorse species assessed by the IUCN Red List, most are described as Data Deficient (26), while others are Vulnerable (10), Least Concerned (1) and Endangered (1) (IUCN, 2014). In recognition of the threats faced by the global trade in seahorses, all species are listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES), which was implemented in May 2004. These species' vulnerability is mostly due to their sparse distribution, low mobility, small home ranges, low fecundity, lengthy parental care and mate fidelity, which make them vulnerable to overfishing and habitat damage (Foster & Vincent, 2004).

Significant declines in seahorse populations (*H. abdominalis*) have been reported in absence of fishing pressure through long term monitoring programs (Martin-Smith & Vincent, 2005). Environmental variables, natural fluctuations and/or recruitment were suggested as possible causes. Likewise, other authors have reported a seasonal fluctuation in the New Holland seahorse's abundance (*H. whitei*) during the breeding season, suggesting that the search for mate could be the driver (Harasti *et al.*, 2012), and population fluctuations have been reported by Masonjones *et al.* (2010) for *H. zosterae* and attributed to seahorse movement patterns. In most cases, no data-supported cause was found for these fluctuations in seahorse abundance.

The Ria Formosa lagoon is a multi-inlet barrier island system and a highly productive lagoon ecosystem sustaining a great variety of commercial species of high economic value (Ribeiro *et al.*, 2006). The use of fishing gears have a direct (by-catch) and indirect (habitat degradation) impact on the local seahorse species (Curtis *et al.*, 2007). In addition, South Portugal is a renowned area for tourism and in the Ria Formosa many human related activities, including aquatic sports, boat traffic and boat anchoring peak during summer.

Clam farming is also a common activity in the lagoon and has led to the removal of natural seagrass, *Zostera noltii,* beds (Guimarães *et al.*, 2012). Harbours and coastal development, combined with dredging to open and maintain navigation channels, have also destroyed vast areas of seahorse habitat (Cunha *et al.*, 2013). Overall, along with anthropogenic activities, natural events are known to alter the condition of seahorses' natural habitat and reduce the availability of holdfasts, essential for seahorse settlement (2010, Correia pers. obs.). The inlets are responsible for the water dynamics within the Ria Formosa and shape the channels and creeks. These areas are particularly vulnerable to the occurrence of periods of high energy waves during winter which leads to severe erosion problems, with frequent overwash of the barrier islands (Martins *et al.,* 1996). These silting events contribute to changes in depth and bottom coverage in certain areas which can have direct impact on local fish communities, including seahorses. Due to the highly dynamic nature of the Ria Formosa lagoon, combined with a temperate climate and variations in seahorse populations reported elsewhere, it is important to assess if any seasonal events might influence seahorse abundance in certain areas within the lagoon. Natural causes such as silting events and sand deposition has contributed to change water flow, shaping the channels and creeks and altering the physical conditions of particular areas in the lagoon (Pacheco *et al.*, 2010)

The first field studies conducted in the Ria Formosa documented the largest seahorse populations ever recorded globally (Curtis & Vincent, 2005). Recent findings however have identified a dramatic decrease in abundance of both seahorse species, *H. guttulatus* (94%) and *H. hippocampus* (73%) (Caldwell & Vincent, 2012) with no clear cause identified. Contributing factors were thought to be changes in habitat, water quality and population fluctuations linked to species interactions.

Although the previous work documenting seahorse populations in the Ria Formosa has highlighted overall trends in abundance of *H. guttulatus* and *H. hippocampus*, it is important to understand the reasons for the observed declines. Also, the seahorse population surveys were done from July to November (Curtis & Vincent, 2005; Caldwell & Vincent, 2012), thus no information is available on seahorse abundance on the remaining months. Therefore, in order to overcome the existing gaps in the seahorse population surveys, this study aimed to quantify the spatial and temporal distribution of the wild populations of the long-snouted

seahorse (*H. guttulatus*) and the short-snouted seahorse (*H. hippocampus*) throughout one year period, and to identify the environmental variables that influences the seahorse population dynamics.

4.3 Material and Methods

4.3.1 Species description

The long-snouted seahorse (*H. guttulatus*) and the short-snouted seahorse (*H. hippocampus*) are two sympatric seahorse species that occur in the Ria Formosa lagoon (South Portugal). These species have distinctive morphological traits including coronet shape, number of trunk rings and skin colour patterns (Lourie *et al.*, 2004). Although these species have overlapping areas of distribution in the Ria Formosa lagoon, they have distinct habitat preferences, as *H. guttulatus* is generally associated with shallow waters and prefers higher habitat complexity, *H. hippocampus* favors deeper areas with lower holdfast availability.

4.3.2 Site description

Underwater surveys were carried out within the Ria Formosa lagoon, south Portugal (36°59'N, 7°51'W). Six locations were chosen based on their different habitat characteristics (Table I) and surveyed on a monthly basis from January to December 2012, i.e., up to 12 survey events per site (Figure 1).

Habitat complexity was considered low if bottom coverage (i.e. number of holdfasts available), was under two holdfasts m^{-2}; medium for 2 to 10 holdfasts m^{-2}; and high for more than 10 holdfasts m^{-2}. Holdfast distribution was considered as patchy when there was a distance between holdfasts in more than a three-meter radius. At each sampling, human related activities were recorded to determine their impact in each site. Sites were considered as highly impacted by human activities when two or more activities were observed in all sampling occasion (i.e., fisheries, boat traffic and anchoring); medium impact when at least two of those activities were observed in at least half of the sampling occasions; and low

impact when less than two activities were observed in less than half of the sampling occasions. A GPS unit was used to accurately determine the location of each study area and during the site delineation. Occasionally sampling was not possible due to poor weather conditions and/or low visibility, thus creating gaps in the monthly scheduled dives.

Figure 4.1: Sites location in the Ria Formosa lagoon. Site 1 (1), Site 2 (2), Site 3 (3), Site 4 (4), Site 5 (5) and Site 6 (6).

Table 4.I: Description of each surveyed site regarding depth (meters); hydrodynamics (water flow); substrate type; habitat complexity (holdfast availability); holdfast distribution; and human impact.

Location	Depth (m)	Hydrodynamics ($m.s^{-1}$)	Substrate	Habitat complexity	Holdfast distribution	Human impact
Site 1	4 – 6	Medium (0.3 - 0.7)	Sand	High	Homogeneous	High (fisheries and boat anchoring)
Site 2	2 - 5	Medium (0.3 - 0.7)	Mud	Low	Patchy	Medium (occasional fisheries)
Site 3	6 - 10	High (0.5 - 1)	Sand	Medium	Patchy	Medium (Boat traffic and occasional fisheries)
Site 4	3 - 5	Medium (0.3 - 0.7)	Mud	High	Homogeneous	Low (no significant human activity)
Site 5	2 - 3	Low (0.1 - 0.5)	Sand/Mud	Medium	Patchy	Low (no human activity)
Site 6	3 - 4	Low (0.1 - 0.5)	Sand	High	Homogeneous	Low (no significant human activity)

4.3.3 Underwater visual census

On each sampling occasion, an underwater visual census technique (Chapter 3) was used. All surveys were conducted at slack high tide for optimal visibility and diminished current. In each survey, seahorses were identified for species, sexed, counted and measured with a ruler and classified into four different size classes, i.e., 0-5cm, 5-10cm, 10-15 cm and 15-20^{+}cm. These four size classes were chosen to classify each individual in four age categories such as 0 to 2 months (0-5cm), 2 to 5 months (5-10cm), 5 to 12 months (10-15cm) and 12^{+} months (15 to 20^{+}cm) for *H. guttulatus*. As for *H. hippocampus* only 3 size classes were considered due to the maximum size that was recorded for this species (<15 cm). Habitat characteristics including substrate type, holdfast availability, depth and water temperature were also recorded.

4.3.4 Statistical analysis – Seahorse density and environment variables

In order to determine the influence of environmental variables on seahorse density, a Spearman rank correlation matrix was used in order to identify correlation between variables and seahorse densities for each species (Zar, 1999). This non-parametric test was used

because environmental variables had non-normal distribution and transformations were unsuccessful. Generalized Linear Models (GLM) were used taking into account the results from Spearman correlation test, using SPSS® v.22 statistical software. As a linear model assumes that the data point comes from a normal distribution, the GLM allows more distributions types rather than a normal one (O'Hara & Kotze, 2010). GLM assumed a negative binomial distribution of the data with deviance as scale parameter method. An Omnibus test was used in order to validate the model accuracy.

4.4 Results

Some surveys could not be completed due to adverse weather conditions during the winter season. Sites 1, 3 and 4 were surveyed in all 12 months, but Site 2 was not surveyed in October and November, site 5 was not surveyed in January, February and September and site 6 was not surveyed in January and September. The data collected from the monthly surveys were used to calculate seahorse abundance (Figure 2), size class distribution (Figure 3a, b) and holdfast usage at first observation (Table II).

4.4.1 Seahorse abundance

An overall total of 1760 seahorses, of which 1675 were *H. guttulatus* and 85 *H. hippocampus*, were found at the six Sites surveyed. After pooling all sites and all monthly surveys, *H. guttulatus* was 20 times more abundant than *H. hippocampus*. The highest *H. guttulatus* abundances were recorded in November at Site 1 (122 seahorses) and Site 4 (72 seahorses), while *H. hippocampus* were observed at highest numbers in May at Site 2 (6 seahorses) and in Site 5 (9 seahorses). No *H. hippocampus* were ever observed at Site 6 in all survey events. First pregnant males, both *H. guttulatus* and *H. hippocampus* were observed in May and the young-of-the year classes were recorded from July onwards in most sites. *H. guttulatus* density ranged from 0.004 (Site 2) to 0.508 seahorse m^{-2} (Site 1) and *H. hippocampus* density from zero to 0.038 seahorse m^{-2} at Site 5. Pooled average seahorse density was 0.107 and 0.005 seahorse m^{-2} for *H. guttulatus* and *H. hippocampus*, respectively.

4.4.2 Seahorse density and environment variables

Spearman correlation tests between environmental variables and seahorse abundance are presented in Table III. Only temperature and depth correlated with *H. guttulatus* abundance. As for the predictive models regarding *H. guttulatus* abundance, the Omnibus test validated the predictive model ($P < 0.001$). Results from GLM indicate that shell fragments seems to be the best predictor for *H. guttulatus* abundance ($P<0.05$) (Table IV). However, bottom coverage of tunicates can be also a predictor for this species, but with barely statistical significance. As for *H. hippocampus*, GLM identified depth as main predictor ($P<0.05$) and tunicates and shells as lower impact predictors.

As for holdfast use, seahorses were found grasping a variety of holdfasts. Holdfast use varied in the different sites surveyed, from shells (Site 1, 5 and 6), tunicates (Site 2, 4 and 5), seagrass (Site 5 and 6) and artificial structures (Site 3). *H. hippocampus* showed no preference for any specific holdfast whereas *H. guttulatus* used shells, tunicates and artificial as preferred holdfast (Table II). Although pooled holdfast use showed a high preference for artificial holdfast (Table II), this result is biased by Site 3. This is an almost barren area and the site's bottom coverage is mainly occupied with a supporting metal chain of an abandoned channel marker buoy.

Water temperature fluctuated from 13ºC in February, up to 24ºC in August (Figure 2). Site 6 was overall warmer than the remaining sites, with water temperatures above 21ºC from April to September.

Fishing activities were mainly observed at Site 1 from March to November. The main fishing gear observed were jigs (used to catch cuttlefish and octopus) at Site 1 and trammel nets at Site 2 and at one occasion at Sites 4, 5 and 6. Boat traffic increased from May to September. Besides recreational boats, transport boats intensified their traffic in the summer, when tourists demand for local beaches is the highest. Boat traffic was higher at Site 1, 2 and 3. Site 2 was highly impacted as it was located close to the main channel and to Olhão's landing pier, where ferry boats base a ferry service between Olhão, Armona and Culatra islands. At Site 1, a large number of sail boats anchored, overlapping the survey area, from July until September.

Table 4.II: Relative abundances (%) of bottom coverage for all sites surveyed.

	Site 1	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**	**Pooled**
Artificial	0.2	13.0	76.8	0.9	0.8	1.8	18.6
Tunicates	15.6	35.9	---	78.6	21.8	7.6	28.6
Barren	1.1	16.3	3.0	0.9	17.3	4.7	3.8
Bryozoan	---	---	1.5	0.9	0.8	2.9	0.9
Codium	1.7	0.0	4.0	---	15.8	3.5	2.9
Rock	---	---	9.7	0.0	---	0.0	2.2
Polychaete	1.3	10.9	0.0	1.1	3.0	5.3	2.0
Seagrass	---	5.4	---	---	13.5	31.8	4.3
Sea Urchin	0.0	---	0.0	0.5	0.0	0.0	0.1
Shell	78.2	17.4	1.2	17.1	15.0	36.5	33.5
Ulva	1.9	1.1	3.7	0.0	12.0	5.9	2.9

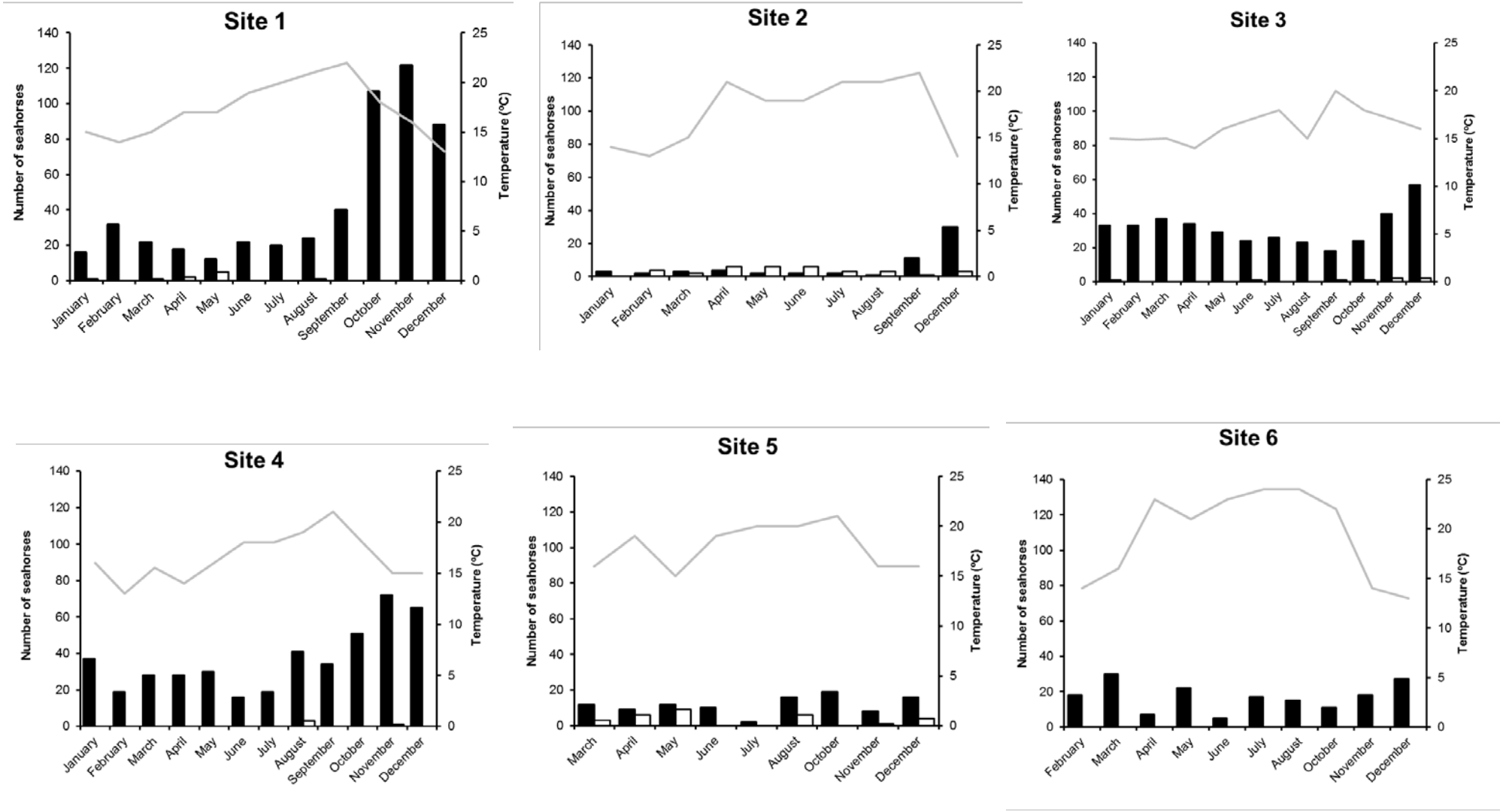

Figure 4.2: Seahorse abundance at Site 1 (a), Site 2 (b), Site 3 (c), Site 4 (d), Site 5 (e) and Site 6 (f), for *H. guttulatus* (■) and *H. hippocampus* (□). Temperature (°C) is shown in 2nd axis.

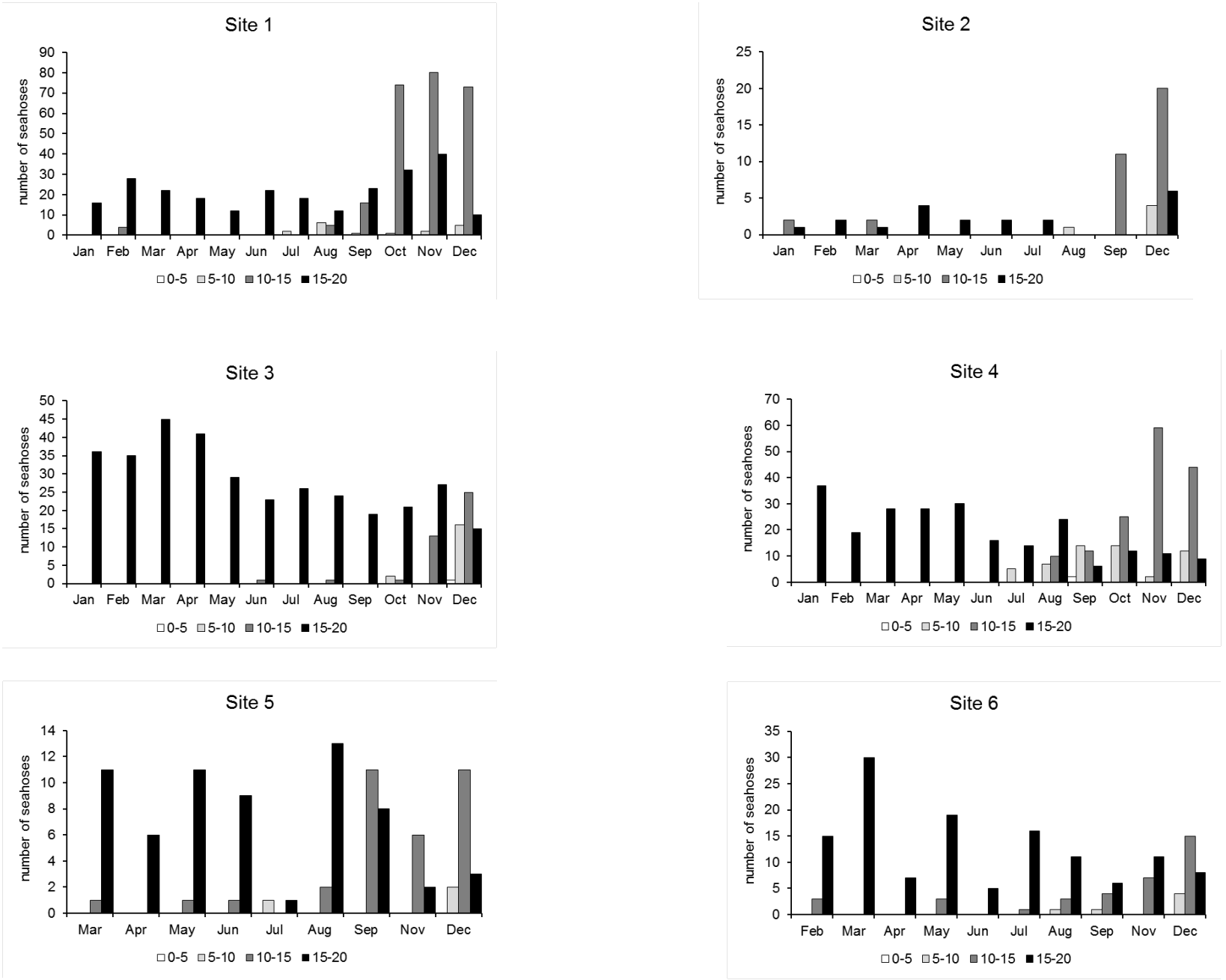

Figure 4.3a: Number of seahorses per size class (cm) of *H. guttulatus* at different sites in all survey events.

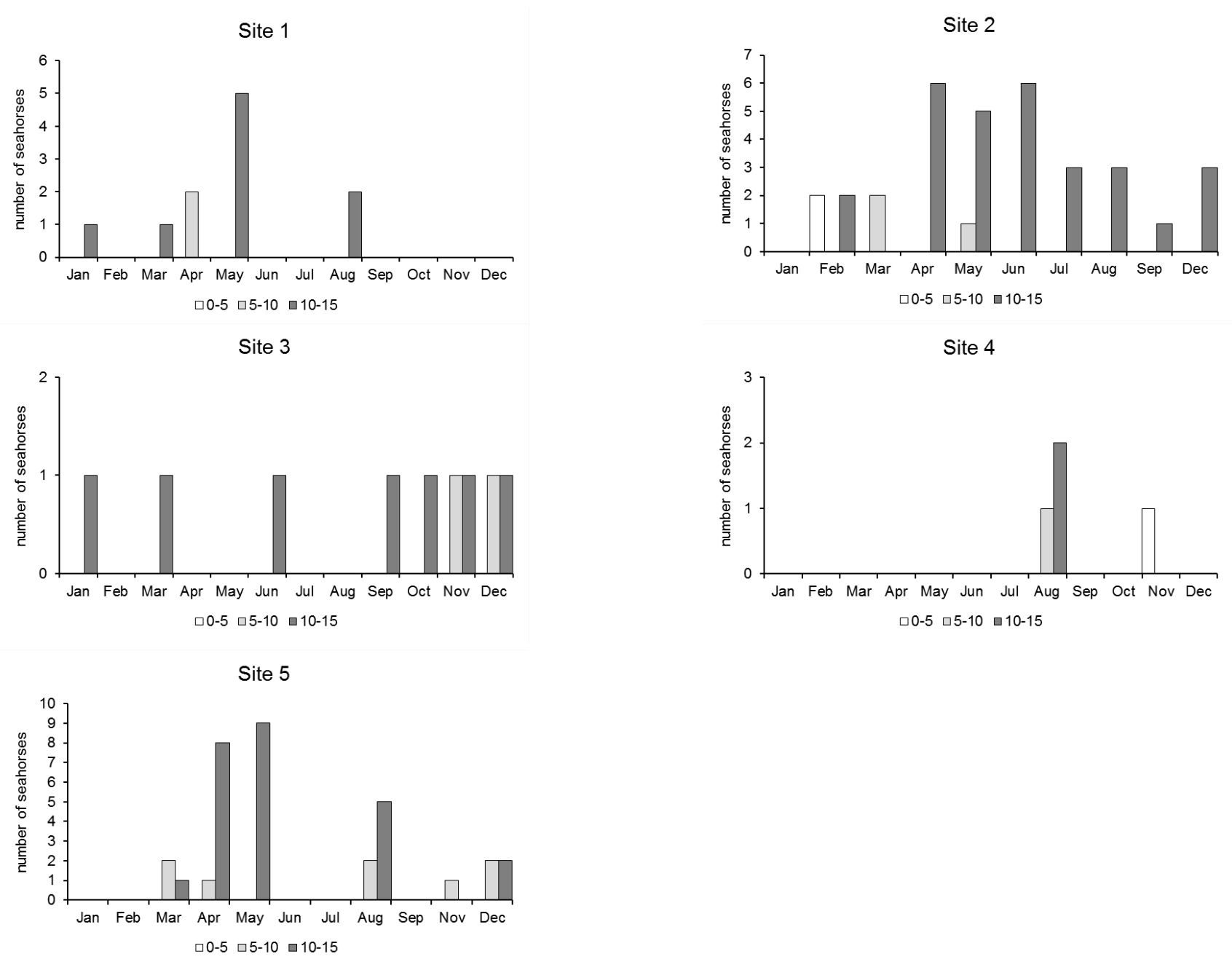

Figure 4.3b: Number of seahorses per size class (cm) of *H. hippocampus* at different sites in all survey events.

Table 4.III: Non-parametric Spearman rank correlation test between seahorse density and habitat variables (data pooled across all sites). Included are habitat variables that were most representative.

Habitat variable	*Hippocampus guttulatus*		*Hippocampus hippocampus*	
	r_s	p	r_s	p
Water temperature	-0.285	0.021	0.143	0.257
Depth	0.417	0.001	0.018	0.889
Holdfast				
% Artificial	-0.019	0.876	-0.058	0.646
% Tunicates	0.116	0.357	0.182	0.146
% Shell	0.060	0.632	0.185	0.140

Table 4.IV: Generalized linear models results for *H. guttulatus* and *H. hippocampus* abundance.

Source	***H. guttulatus***		***H. hippocampus***	
	Wald Chi-Square	Sig.	Wald Chi-Square	Sig.
Temperature	1.575	0.209	2.002	0.157
Depth	1.601	0.206	6.029	0.014*
Percent_Artificial	1.043	0.307	3.437	0.064**
Percent_Tunicates	3.220	0.073**	0.139	0.709
Percent_Shell	5.195	0.023*	2.825	0.093**

* Significant with 95% interval confidence

** Significant with 90% interval confidence

4.5 Discussion

In this study, six sites were chosen based on previous surveyed locations, so that enough data were available to monitor whether there were seasonal changes in seahorse abundance. Sites were chosen due to their characteristics and location thus representing the range of different environmental variables that occur in the Ria, and also in consideration to previous studies (Curtis & Vincent, 2005; Caldwell & Vincent, 2012). However, as the selected sites seemed to be representative of most seahorse habitats, results can help to shed some light over the changes and events responsible for seahorse abundance fluctuations, but the seahorse densities reported for each site cannot be used to infer an overall seahorse abundance in all the Ria Formosa. Previous studies on

seahorse abundance reported mean densities ranging from 0.001 to 0.330 seahorse m^{-2} (Table V). Considering that the aim of this study was focused on the seahorse population seasonal dynamics, the sites selected for this study were chosen based on seahorse presence, however, with no minimum threshold of seahorses per site. Thus, comparing the obtained results with previous studies there was an apparent increase in *H. guttulatus* numbers, but a long term monitoring program should be carried out in order to document the seahorse population trend in the Ria Formosa (Chapter 2).

Table 4.V: Seahorse species densities in previous studies.

Species	Density (seahorse.m^{-2})	Location	Year	Author(s)
Hippocampus abdominalis	0.010	Derwent estuary, Australia	2001-2014	(Martin-Smith & Vincent, 2005)
Hippocampus reidi	0.026	Brazil	2002-2006	(Rosa et al., 2007)
Hippocampus reidi	0.180	Ilha Grande, Brazil	2002-2004	(Freret-Meurer & Andreata, 2008)
Hippocampus zosterae	0.080	Tampa Bay, USA	2005-2007	(Masonjones et al., 2010)
Hippocampus capensis	0.330	Knysna Estuary, South Africa	2001	(Teske et al., 2007)
Hippocampus guttulatus	0.018	Mar Piccolo di Taranto, Italy	2011	(Gristina et al., 2014)
Hippocampus guttulatus	0.073	Ria Formosa, Portugal	2001-2002	(Curtis & Vincent, 2005)
Hippocampus guttulatus	0.004	Ria Formosa, Portugal	2008-2009	(Caldwell & Vincent, 2012)
Hippocampus guttulatus	0.107	Ria Formosa, Portugal	2012	Current study
Hippocampus hippocampus	0.007	Ria Formosa, Portugal	2001-2002	(Curtis & Vincent, 2005)
Hippocampus hippocampus	0.001	Ria Formosa, Portugal	2008-2009	(Caldwell & Vincent, 2012)
Hippocampus hippocampus	0.005	Ria Formosa, Portugal	2012	Current study

The higher overall abundance of *H. guttulatus* was observed in sites with high holdfast availability (i.e. Sites 1, 3 and 4), while the highest *H. hippocampus* abundance was recorded in sites with medium to low holdfast availability and muddy substrate (i.e., Sites 2 and 5). These observations agree with Curtis & Vincent (2005) who reported a higher affinity to sandy bottom and higher complexity habitats for *H. guttulatus*, while *H. hippocampus* seemed to prefer habitats of less complexity and muddy substrate.

H. guttulatus density correlated positively with depth and negatively with temperature. This fact agrees with the findings of Caldwell & Vincent (2012) and might explain the positive correlation between depth and *H. guttulatus* density. During the surveys depth

ranged from 2 to 10 meters and higher *H. guttulatus* abundances were found in the range of 4 to 6 meters depth. Moreover, areas of just 2 meters depth at high tide can be considered as intertidal zones and therefore unsuitable for seahorse settlement. The negative correlation found between temperature and *H. guttulatus* might be related as seasonal related factor rather than the variable itself. In fact, in the summer months there was an overall decrease in seahorse density, when the temperature tend to be higher. Temperature was overall higher at Site 6 probably due to a combined factor of low depth and low water flow. Although this site has high holdfast availability and low human impact, which should favor *H. guttulatus* abundance (Chapter 2), the temperature might be the driver for seahorse dispersal. In fact, Palma *et al.* (2012) suggests that the optimal temperature for improved growth rate of *H. guttulatus* in captivity occurs between 20 to 21ºC.

Statistically, there was no correlation between environmental data and *H. hippocampus* density, a finding that reduces the theoretical predictability of the abundance of this species. However, in this study, this might be influenced by the low abundance recorded during the surveys, and notably their absence in several survey occasions, particullarly at Site 6. Higher *H. hippocampus* densities were found in Site 2 and Site 5. These sites have the low to medium habitat complexity with a muddy substrate, reported by Curtis & Vincent (2005) as favorable habitat for this species. From all the variables included in the GLM, habitat variables seemed to be the best predictors to estimate seahorse abundance. In addition, *H. hippocampus* abundance seemed to have depth as good predictor, which agrees with Curtis & Vincent (2005),.

H. guttulatus abundance decreased in the months of May until July and then increased from August to December, particularly in Sites 1, 3 and 4 where highest densities were observed. This decrease in abundance happened during the species' breeding season, when *H. guttulatus* and *H. hippocampus* pregnant males were observed (from May to late August which agrees with Lourie et al. (2004)) suggesting that the search for mate could be the driver. In fact, the home range reported for *H. guttulatus* was 20 m^2 (Curtis & Vincent, 2006) but they have been observed travelling longer distances (Caldwell & Vincent, 2013) which might suggest that they can expand their home range due to environmental factors, reproductive behaviour or unsuitable habitat. As fewer *H. hippocampus* were sighted, it was not possible to find any clear fluctuation in that species' abundance. The young-of-the-year classes were observed from July onwards wich

suggests a new recruitment to those areas, contributing to the increase in seahorse abundance. Following the same pattern as the adults, the younger *H. guttulatus* seem to recruit not only to areas with high habitat complexity, but also to locations where the species is more abundant. In fact, these areas may benefit the young seahorses during the first stages of developments, as they can provide them improved protection due to enhanced holdfast availability and a more reliable food resource.

In this study, site 5 is located next to the Ancão's inlet, which makes it more vulnerable to silting events. During January and February, winter storms were responsible for physical changes in the area creating a silting event in the inlet that lead to an decrease in the local water dynamics. This reduction in the water flow promoted a progressive sand deposition causing a direct impact in the seagrass bed coverage. Roughly 17% of the seahorses found at Site 5 were located in barren areas, i.e, not grasping any holdfast. This percentage may be due to the progressively diminished holdfast availability, distribution and low current which combined forced seahorse movement in search of nearby holdfasts. Although seahorses are known as limited swimmers and sedentary fish, they can adapt to progressive habitat changes by moving to more suitable areas (Masonjones *et al.*, 2010). Nevertheless, this dispersal might fragment local populations and have a direct impact on relative reproduction success and therefore affecting that year's recuitment and ultimately the seahorse overall abundance.

Fisheries activity has also been observed, with higher incidence in Site 1, in 9 of the 12 surveys, an area closely located to the fishing village of Culatra. Here, fishermen use a wide array of fishing gears including traps, jigs, gill and trammel nets. The use of trammel nets has a direct impact on the local seahorse populations, as the seahorses get tangled in the net and when hauled on board they are not returned back (Correia, pers. obs.). In addition, there has been information from authorities of an increase in illegal bottom trawling fisheries activity. This highly invasive fishing method is widely recognized as having deletrious impact on bottom habitat structure (Auster *et al.*, 1996; Thrush & Dayton, 2002a; Althaus *et al.*, 2009) thus leading to its degradation and consequently affecting the local fish assemblage, in particular benthic dwelling seahorse species (Scales, 2010; Vincent *et al.*, 2011).

The Ria Formosa lagoon is generally known as a popular area for tourism and in the summer, human related activities as aquatic sports, fishing and boat traffic greatly

increases. In fact, traffic is increased by either recreational boating, boat transfers and taxis that transport people to the nearby beaches. Also, sail boats widely use the overlapping area of Site 1 as a mooring site from May to September (Correia, pers. obs.). These combined activities add pressure to the local seahorse populations causing seahorse dispersal. Site 2 is also higly impacted by boat traffic as it is located close to Olhão's landing pier and close to the ferry transfer route between Olhão and Armona. Although no direct impact on seahorse populations has been reported from boat traffic, authors have reported that dredges done by boat propellers on seagrass beds might be an indirect cause for habitat degradation (Bell *et al.*, 2002; Hardiman & Burgin, 2010). In addition, seahorses have been proven to be sensitive to chronic sound wich might lead to weight loss and overall health deterioration (Anderson *et al.*, 2011).

Higher *H. guttulatus* density (0.51 seahorse m^{-2}) was reported in November 2012 at Site 1. This density is similar to the highest density reported in 2002 by (Curtis & Vincent (2005)). Nevertheless, lower seahorse densities were observed at the same site in different months (0.05 seahorse m^{-2} in May 2012). This fact highlights the importance of an extended monitoring effort in order to avoid bias due to population density seasonal fluctuation. Other factors such as water current and holdfast distribution might influence the distribution of the seahorses in a particular area. Thus, higher water flows might promote seahorse holdfast grasping whereas lower water current situations might allow higher seahorse movement and dispersal. These are important aspects to consider when doing a seahorse population census in a particular area.

In conclusion, this study suggests that seasonal events might be responsible for fluctuations, especially in areas where significant pressure from human activities exists. Also, as is case of the Ria Formosa lagoon, highly dynamic areas might be more prone to physical habitat changes that might lead to benthic species abundance shifts.

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Chapter 5. The use of a non-invasive tool for capture-recapture studies on a seahorse (*Hippocampus guttulatus*) population

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5.1 Abstract

Photo-identification is a non-invasive technique that has been successfully used to study population parameters in Syngnathids. In this study, the *Hippocampus guttulatus* spot pattern was analyzed using a computer program algorithm which allowed individual comparison. This methodology was firstly tested in controlled environment using 51 adults (F2 generation) and 55 juvenile seahorses (F3 generation). Positive matches were obtained in 86.3% and 83.6% of the adults and juveniles, respectively. In a second experiment, monthly surveys were carried out in 5 selected locations in the Ria Formosa lagoon, South Portugal, over the course of a year and total of 980 pictures were analyzed. Photographed fish were re-sighted 1 to 9 times during the course of the survey period with an overall re-sight record of over 30%. Photo-identification was therefore shown to be a useful tool for non-invasive mark-recapture studies that can be successfully used to survey the population abundance of *H. guttulatus* aged 6 months or older in consecutive years. This could be of great value when considering the assessment of *H. guttulatus* populations and understanding changes over time.

Keywords: *Hippocampus guttulatus*; Photo-identification; mark-recapture; population abundance

5.2 Introduction

Estimates of population size, survival, reproduction and movement rates using capture–recapture models require the ability to identify previously marked or sighted individual animals (Nichols, 1992). Individual identification is important in conservation studies when considering the species distribution, habitat use and population status (Williams *et al.*, 2002). This will allow to track individual movements and model population estimates. Tags have been used as a tool for studies in fish ecology with a wide variety of applications (Parker *et al.*, 1992; Winner *et al.*, 1999; Baras *et al.*, 2000; Pine *et al.*, 2003; Holm *et al.*, 2007; Hutson *et al.*, 2007; Jiang *et al.*, 2007). The use of artificial tags, either internal or external, is widely recognized as an effective method of marking animals but have been associated with physical and behavioral disruptions (Mellas & Haynes, 1985; Marty & Summerfelt, 1986; Moore *et al.*, 1990; Murray & Fuller, 2000; Welch *et al.*, 2007). Ideally, tags should have minimal effects on mortality, growth and reproduction of the target species so that the results from tagging studies may accurately reflect the parameters of the study population (Willis & Babcock, 1998). Nevertheless, methods for identifying individual animals that rely on artificial markings such as neck collars, transponders, tissue removal, dyes and chemical markers, can be categorized as invasive (Silvy *et al.*, 2005). The most common used artificial tags in the Syngnathidae family (seahorses, pipefish and seadragons) are external tags (necklaces) and internal tags (Visible Implant Fluorescent Elastomer – VIFE) (Monteiro *et al.*, 2005; Curtis, 2006b; Sogabe *et al.*, 2007; Palma *et al.*, 2008; Caldwell *et al.*, 2011; Harasti *et al.*, 2012). VIFE tags have been used as a valuable tool in fisheries research and management (FitzGerald *et al.*, 2004) because they are cost-effective, have low rates of tag loss, and have negligible effects on survival, growth, and behavior (Willis & Babcock, 1998; Willis *et al.*, 2001; Goldsmith *et al.*, 2003; Woods & Martin-Smith, 2004; Curtis, 2006b). Lately, the use electronic tags have been proven to be an effective method of collecting data on individuals of *Hippocampus guttulatus*, although over a shorter time period (Caldwell et al., 2011).

Natural marks or patterns can provide an alternative method of individual identification provided that there is enough polymorphism and information content in the characteristic in question (Anderson *et al.*, 2007). The use of natural marks using photo-identification is a non-invasive technique and has been successfully used to study different marine species (Würsig & Jefferson, 1992; Meekan *et al.*, 2006; Van Tienhoven *et al.*, 2007;

Barker & Williamson, 2010; Kitchen-Wheeler, 2010) including syngnathids (Martin-Smith, 2011). In addition to enabling individual identification, the use of natural marks in capture-recapture studies must comply with several requirements: Markings must remain consistent over time and identifiable in order to be usefully used as an estimate tool for studying population variables (Anderson *et al.*, 2007; Martin-Smith, 2011). In fact, several studies have reported that these markings may persist well over 10 years after its initial recording in species such as *Carcharias taurus* (Bansemer & Bennett, 2008), *Stegostoma fasciatum* (Dudgeon *et al.*, 2008) and *Phyllopteryx taeniolatus* (Martin-Smith, 2011).

The long-snouted seahorse, *H. guttulatus*, is a European species which occurs in the Ria Formosa lagoon, South Portugal. The greatest population size recorded for this species throughout its range was recorded in the early 2000's (Curtis & Vincent, 2005), however recent field data showed a significant decrease of 94% in the seahorse populations within this lagoon (Caldwell & Vincent, 2012). The use of non-invasive techniques for seahorse monitoring is of paramount importance when considering the study of these dwindling populations. The long-snouted seahorse has a distinct mark pattern composed of white spots scattered throughout the entire body surface. The aim of this study was to evaluate the potential of using photo-identification as a monitoring tool when surveying *H. guttulatus* populations.

5.3 Material and Methods

5.3.1 Preliminary experiment – Controlled environment

A total of 51 adult *H. guttulatus* (24 females and 27 males; F2 generations) and 55 juveniles (30 females and 25 males; F3 generation) were used in this preliminary trial. Seahorses were reared and kept at the Aquaculture station of the Centro de Ciências do Mar and separated according to their generation in 250L tanks, in a flow-through system, with moderate aeration and fed on frozen shrimp (*Palaemonetes varians*). At the start of the experiment, adults and juveniles were aged 18 and 6 month old, respectively. Adults averaged 17.5±1.8 cm total length while juveniles measured 12.3±0.9 cm.

On the photographic recording day, animals were removed from their culture tanks and individually placed in a clear 10L glass tank. Each seahorses was then gently placed close to the aquarium's wall with their left side facing the glass to allow full visibility and thus optimize the photos quality. After these adjustments were made, a digital camera (Sealife® DC1200) was used to photograph each animal.

A second round of photo were undertaken 2 months later, using the exact same animals and protocol as before, in a random order. Again, photos were taken of the seahorses' left side for consistency. This procedure was used as a control method to determine the viability and accuracy of the photo-identification on this species as in this case, the same animals were used in both photo sessions under controlled conditions.

5.3.2 *In situ* observations

This experiment was conducted at 5 sites (Site#1 to 5) (Figure 1) in the Ria Formosa lagoon, South Portugal (36º59´N, 7º51´W). Sites were surveyed on a monthly basis over a 12 month period (2012). Each month, in each sampling site, a modified Underwater Visual Census (UVC) technique (Chapter 3), based on the UVC previously used by Caldwell & Vincent (2012), was used to survey an area of 240m^2 (30x8m). On some occasions sampling was not possible due to poor weather conditions and/or low visibility, thus creating gaps in our monthly scheduled dives. A GPS unit was used to determine the locations of each study area and during the site delineation the same bearing was taken while laying each transect so that the same area could be consistently covered on each sampling occasion. Each site differed in its habitat complexity (Table I).

Table 5.I: Site description considering substrate type, depth and habitat complexity.

	Substrate	Depth (meters)	Habitat complexity
Site#1	Sandy	5 to 6	high: mostly shells and sessile invertrebrates (*Ascidia* sp., *Sabella* sp., sea urchins)
Site#2	Sandy	6 to 9	low: scattered rocks, mostly barren
Site#3	Muddy	3 to 5	high: *Ascidia* sp. and shells
Site#4	Muddy	2 to 3	high: seagrass bed, shells and *Sabella* sp. tubes

Site#5	Sandy	2 to 3	low: scattered *Codium* sp., and small seagrass patches

Figure 5.1: Location of the survey sites (Site 1-5) in the Ria Formosa lagoon, South Portugal.

During each survey, seahorses found was counted, sexed and photographed on its left side using the same underwater camera (Sealife® DC1200) as in the *ex situ* trials. When possible, photos were taken with no direct interaction with the seahorses. However, when this was not possible, the seahorses were gently handled for the minimum time possible to reduce stress from the procedure. As the surveys were done over a 1 year period, only adults aged 1+ year old were photographed and all juveniles were discarded. Seahorse were measured according to (Lourie *et al.*, 1999b), and those that were at least 15 cm in total length were considered as 1+ year old adults, as suggested by Curtis & Vincent (2006). Although laboratory results indicated that photo-identification methodology could potentially be used on juveniles older than 6 months, this study only focused on the breeding population. As aging juvenile seahorses can be very subjective, we removed this bias by only working on larger seahorses considered to be fully adult.

5.3.3 Image Analysis

Photos were downloaded and computer labeled to include the individual/photograph number sex, date and site (*in situ*) information. In this study, it was decided to focus on analyzing the spot pattern in the head of each fish for individual identification (Figure 2).

As the spots were clearly distinguishable no further photo adjustments were necessary. Each spot pattern was recorded using the computer software algorithm, I3S® Manta v. 2.1 and analyzed to determine eventual matches.

Figure 5.2: Selected area for spot marking in I3SM® Manta software with 3 reference points (A – eye ball, B – upper origin of the pectoral fin, and C – lower origin of the pectoral fin).

The identification procedure assumes that the spot pattern of each individual animal is a unique distinguishing feature. The user points out the most distinguishing spots of each image and draws an ellipse around each spot after choosing 3 landmark points on each image, i.e., the center of the eye ball and the upper and lower limit of the pectoral fin. This information will provide relative position and size of each spot. This spot pattern is then stored in a 'fingerprint file'. The program's algorithm (Van Tienhoven *et al.*, 2007) will then process each file providing the user a ranked list of possible matches. The final decision on a true match is left to the observer through image visual comparison. Each comparison batch was done using all photos recorded in captivity (preliminary experiment) or same sampling site (*in situ*). Considering that each site was distant from each other of more than 1km and low mobility and the sedentary nature of this species of seahorse previously recorded at this location (Curtis & Vincent, 2006), the probability of

matches between different sampling sites were considered to be very limited. Also, the photos were divided by sex prior to comparison as this allowed a smaller batch of pictures to be compared and therefore increase the possibility of finding matches. The list of possible matches was then analyzed and a visual comparison was made to confirm each match.

5.3.4 Wild Population Parameters

To estimate population abundance at each site, and considering the annual recruitment period of this species reported by Curtis & Vincent (2006), a Cormack-Jolly-Seber (CJS) open model population was used (Cormack, 1964; Jolly, 1965; Seber, 1965). This model assumes that: Animals retain their tags throughout the experiment and that tags are read properly; catchability is constant for all animals (marked and unmarked) for all sampling periods; sampling periods are instantaneous and recaptured animals are released immediately. Abundance ($\hat{N}$), capture probability (p) and apparent survival (ϕ) parameters were determined using a CJS open population model in the MARK software (White & Burnham, 1999). Finally a "program CAPTURE" goodness of fit test was run inside the MARK software to test if the model adequately fits the data. In cases of low recapture data, a cumulative non-linear model ($y=ax/(b+x)$) was used to fit the data and estimate abundance according to Kohn *et al.* (1999).

5.4 Results

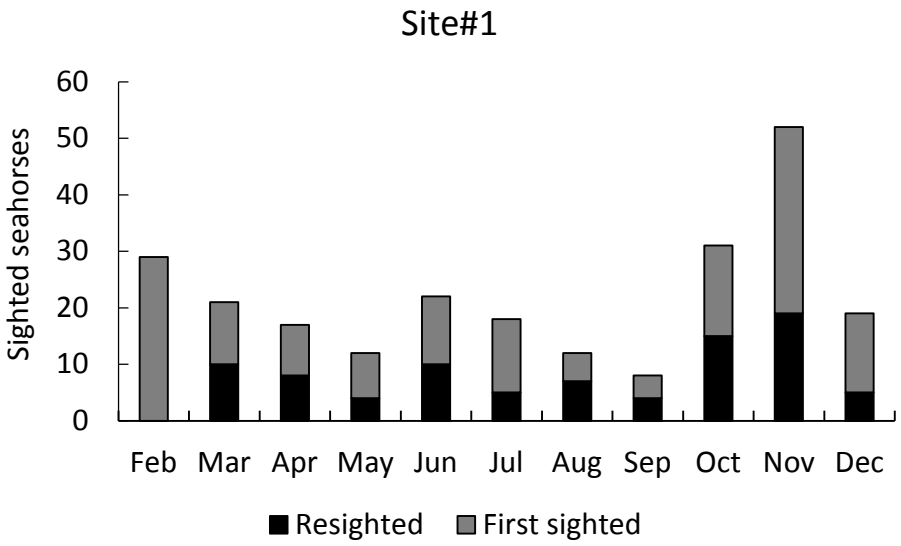
5.4.1 Preliminary experiment – controlled environment

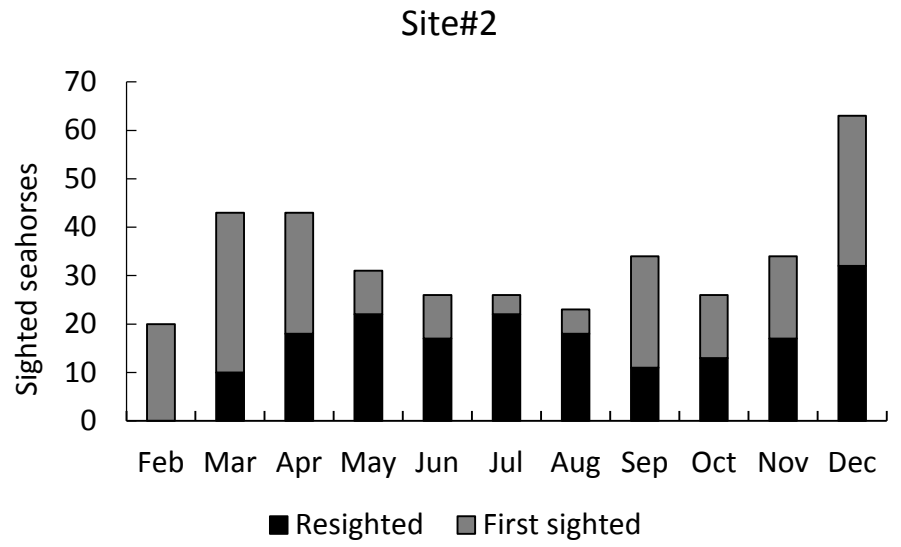
Using the I3S Manta® software, there was a high matching accuracy when comparing the two sets of photos. From the 212 photos analyzed, positive matches were classified as first rank in 86.3% for adult (84.0% males and 83.3% females) and 83.6% for juveniles (92.6% males and 79.2% females) in the overall output rank list. Inconclusive matches that were classified as second rank or below were caused by poor photo quality, incorrect positioning of the seahorse, or even due to algorithm errors. These inconclusive matches were then analyzed by direct visual comparison and a corresponding positive match was found.

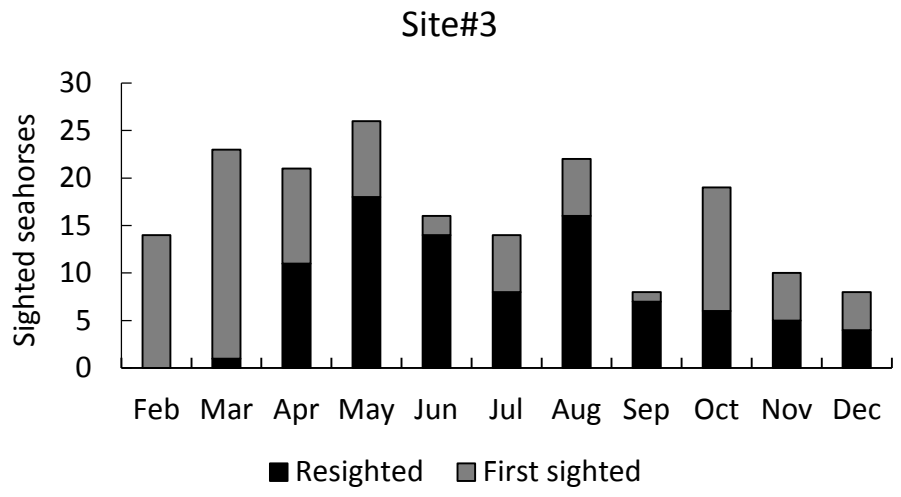
There were no statistical differences related to gender either in adults or juvenile seahorses (Fisher's exact test, d.f. = 1; P>0.05). The number of spots varied between each individual from 37 to 71 averaging 53.7±9.8 and 48.1±5.9 for males and females, respectively. No statistical differences were found in number of spots between males and females (χ^2 =25.2, d.f.=22; P=0.287).

5.4.2 *In situ*

A total of 980 photos were analyzed in all sites surveyed, 217 in Site#1, 363 in Site#2, 181 in Site#3, 134 in Site#4 and 85 in Site#5. Recapture percentage, i.e, percentage of animals resighted at least once in a sampling period, varied from a minimum of 13.6% in Site#4 to a maximum of 44% in Site#3, with an overall average of 31.3±13.1% of the total sighted animals at all 5 sites (Figure 3). Total unique individuals found during the sampling period was 131 for Site#1, 209 for Site#2, 91 for Site#3, 110 for Site#4 and 65 for Site#5.


Site#1
Sighted seahorses
60
50
40
30
20
10
0
Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec
Resighted
First sighted


Site#2
Sighted seahorses
70
60
50
40
30
20
10
0
Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec
Resighted
First sighted


Site#3
Sighted seahorses
30
25
20
15
10
5
0
Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec
Resighted
First sighted

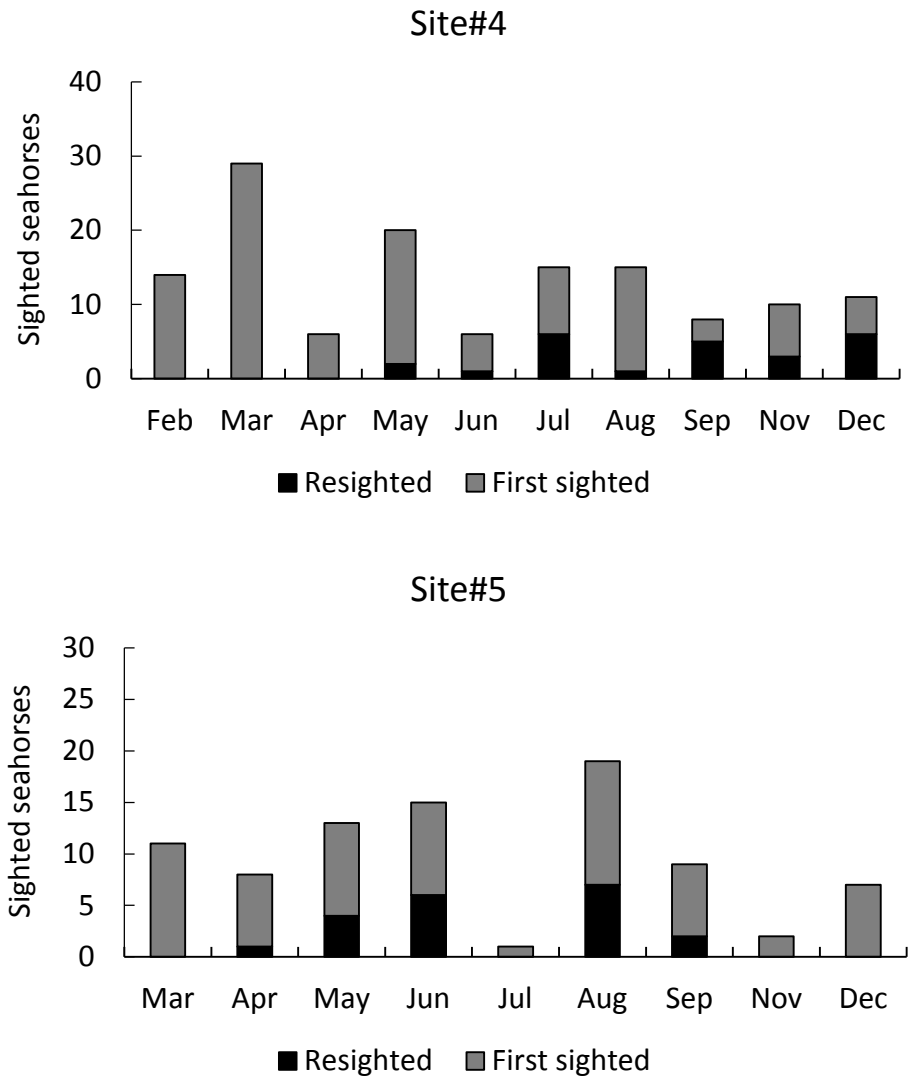


Figure 5.3: Resight history for each site surveyed on a monthly basis throughout the experiment.

A total of 980 photographs were recorded in all sites (pooled value for first-sighted and resighted fishes). Overall average seahorse densities (considering the full sampling period) were of 0.091±0.051, 0.140±0.051, 0.069±0.026, 0.056±0.029 and 0.039±0.024 seahorse m^{-2} for Site#1 to 5 respectively; with maximum densities varying between 0.004 (Site#5) and 0.263 seahorse m^{-2} (Site#2).

Most resighted seahorses were only found in one subsequent sampling occasion (69.1±11.2%), while the remainder were resighted in 2 to 9 surveying events. Overall relative number of resights per site is presented in Figure 4.

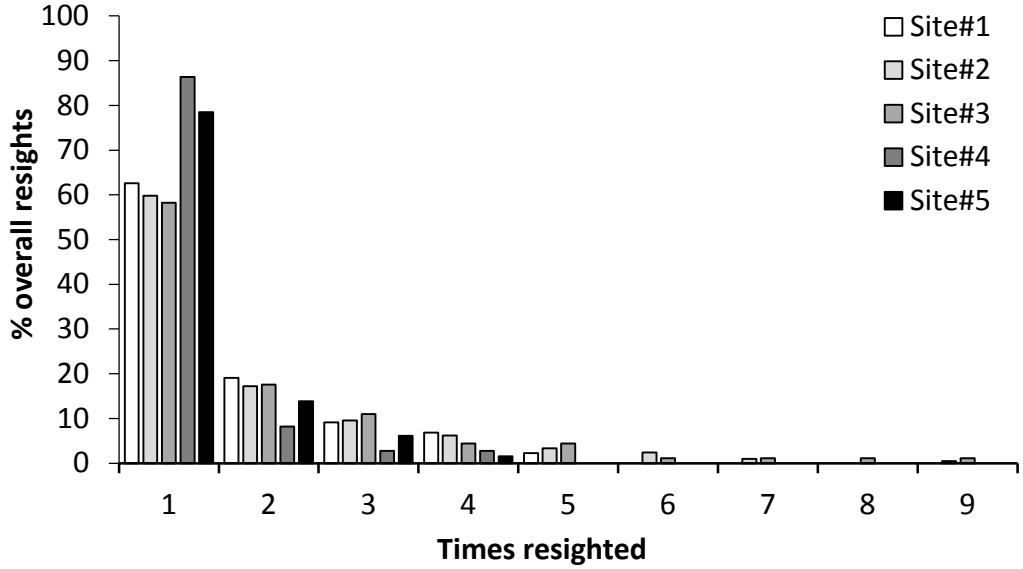


Figure 5.4: Overall relative resight history for each site surveyed considering all photographed seahorse.

5.4.3 Wild Population Parameters

Due to low recapture data in Site#4, abundance was estimated using the cumulative non-linear model. Abundance estimates varied from 108 (Site#3) to 257 (Site#2) (Table II).

Table 5.II: Abundance estimate ($\hat{N}$) obtained by POPAN model, apparent survival (φ) and resight probability ($\hat{p}$) for each site were obtained using Cormack-Jolly-Seber open population model and Cumulative curve model (y=ax/(b+x)).

CJS Open Population model							
	$\hat{N}$	Φ	CI (95%)		$\hat{p}$	CI (95%)	
Site#1	131	0.92	0.83	0.97	0.19	0.10	0.33
Site#2	209	0.82	0.52	0.91	0.40	0.25	0.57
Site#3	91	0.71	0.51	0.87	0.46	0.37	0.55
Site#5	65	0.89	0.43	0.99	0.19	0.05	0.48
Cumulative Curve							
	$\hat{N}$	95% CI		R^2			
Site#4	217	166	267	0.9866			

5.5 Discussion

The results obtained in the preliminary *ex situ* experiment fully supported the photo-identification as an individual non-invasive marking technique that is applicable to the long-snouted seahorse, *H. guttulatus*. Photo-identification has been successfully used as a mark-recapture technique in many studies and has proven to be an effective non-invasive technique for larger marine species such as *Balaena mysticetus* (Rugh *et al.*, 1992), *Ursus maritimus* (Anderson *et al.*, 2007), *Globicephala melas* (Auger-Méthé & Whitehead, 2007), *Carcharias taurus* (Bansemer & Bennett, 2008) and *Rhincodon typus* (Holmberg *et al.*, 2009). In addition, photo-identification has been used to obtain accurate data in mark-recapture studies in syngnathid species such as *Phycodurus eques* (Connolly et al., 2002), *Nerophis lumbriciformis* (Monteiro *et al.*, 2005),*Phyllopteryx taeniolatus* (Martin-Smith, 2011) and *Hippocampus reidi* (Freret-Meurer *et al.*, 2013). This technique uses very little or no handling thus causing reduced stress in the wild populations studied when compared to other methods. From previous seahorse tagging techniques, VIFE appears to be the most widely used and proven to be effective for individually identifying wild seahorses (Le Cheminant, 2000; Morgan & Martin-Smith, 2004; Sanchez-Camara & Booth, 2004; Woods & Martin-Smith, 2004). However, this methodology has some implications for animal welfare as it involves handling and ink injection individuals during a long period of time (Curtis, 2006b). In addition, the VIFE must be purchased and takes time to apply, therefore requiring more resources.

The I3SM® Manta v2.1 software used in this study has already proven to be effective in mark-recapture studies for other species, including sharks (Meekan *et al.*, 2006; Wilson *et al.*, 2006; Bradshaw *et al.*, 2007), manta rays (Marshall *et al.*, 2008), polar bears (Anderson *et al.*, 2007) and seadragons (Martin-Smith, 2011) with high accuracy results. The correct contour of the spots is crucial for an optimal identification accuracy. Also, in some cases the shape of the spots are not perfectly elliptical nor circular thus creating the potential for some variation due to each user criteria while selecting the spots within the program I3SM® Manta. Therefore, as in other sampling methods, data should be processed by as few researchers as possible to minimize bias. In the present study, this process was done by only one user, the criteria for the selection of each spot remained consistent and was confirmed by the high match percentages obtained when processing the photographs of the animals in controlled conditions, similar to those obtained by Martin-Smith (2011) with *Phyllopteryx taeniolatus*. Another equally important detail to

consider to the success of this methodology is the photo quality of the target animal. Underwater photography in the Ria Formosa can be difficult due to low visibility. Visibility in most dives varied from 0.5 to 5 meters, but in most cases was recorded to be less than 2 meters which has an impact on the final quality of the resulting pictures, making it hard to identify each spot in some occasions. Also, as observed in the preliminary *ex situ* experiments, the seahorse should be ideally positioned parallel to the camera in order to get a straight profile picture. Even though it was necessary to handle each seahorse to make the photo, the handling was done using gloves and each seahorse was gently gripped to avoid skin injuries and handling time was kept to the minimum possible, and was only a few seconds per animal. Where photos were blurred, the software I3SM® Manta was ineffective and each photo then needed to be validated by visual comparison. In fact, visual comparison was a good means to check individual identifications, in addition to the above mentioned cases where photograph quality was poor. In these cases, the researcher may look for other distinctive marks or spots that could add extra information to help to decide possible matches.

The present study also proved that photo-identification is also valid when used in juveniles aged 6 to 8 months old, suggesting that the spot pattern and shape have low or negligible modifications with age. This result agrees with Martin-Smith (2011) that found *Phyllopteryx taeniolatus* to have a unique spot pattern that did not undergo significant changes over a period of at least 18 months. When considering tagging methods for mark–recapture studies, researchers must verify if the tags in use comply with the assumptions of their mark–recapture models. For open population models, those assumptions include that tagged fish are a random sample of the population of interest; numbers of releases are known; tagging is accurate, no tag loss or misread tags; Animals are released within a brief time period; the fate of individual fish and the fates of fish in differing cohorts are independent (Jolly, 1965; Seber, 1965). The body area chosen for identification purposes seemed to be adequate as it allowed the correct identification of each individual and with no gender variation and no significant changes were observed in spots shape and pattern in all animals photographed, during the entire survey period. However, considering that the spot pattern is present throughout the *H. guttulatus* body, the area of focus could be expanded in order to increase the matching accuracy.

Overall, roughly one third (31.3±13.1%) of all photographed animals were resighted. This result suggests a sedentary behavior of this species, when compared with other migrant

fish species (Templeman, 1984; McGovern *et al.*, 2005), and agrees with their well-documented small home range (Curtis & Vincent, 2005; 2006; Caldwell *et al.*, 2011). Several animals were resighted in multiple occasions throughout the year up to 9 out of 11 in the monthly surveys. Although Curtis & Vincent (2006) reported that home-range for this species after settlement was approximately 20m, *H. guttulatus* have a patchy distribution that may be conditioned by the level of habitat complexity and population density (Chapter 2). Caldwell & Vincent (2013) reported that *H. guttulatus* can move more than 100m from its original location during a relative short period of time and display an individual habitat preference. This behavior could explain the absence in the survey area of some resighted seahorses in specific months, as they might adjust their location depending on holdfast and food availability, seahorse density, fishing activities and weather conditions. Nevertheless, the area covered in each survey ($240m^2$) seems to be adequate as it overlaps the equivalent of several home ranges reported for this species (Curtis & Vincent, 2006; Garrick-Maidment *et al.*, 2011; Caldwell & Vincent, 2013). After the completion of the monthly surveys, the individual resight occasions were approximately of 20% and 10% for animals resighted 2 and 3 times respectively. This could suggest that even though seahorses have small home ranges, they can do small migration movements, returning to the original area. This behavior was observed during the tidal peaks when the current is weak and fish undertake the hunting and feeding behavior. The highest resight number in this study was registered in Site#2 which could be due to the nature of its habitat characteristics. This area is very hydrodynamic has low habitat complexity and the available holdfast is only provided by an abandoned metal chain from an old signaling buoy that is laid straight on the bottom, in an otherwise barren area of sandy substrate. This structure provides the only holdfast for seahorses which is necessary to cope with the high water currents that occur during the high tide to low tide transition period. This low complexity might cause a sedentary behavior on the local seahorse population and therefore increasing the resight occasions.

Seahorse highest density was reported in Site#5 (0.263 seahorse m^{-2}) and was well above those reported by Caldwell & Vincent (2012) which was of 0.004 seahorse m^{-2} on average, considering the 33 sites surveyed, and with maximum density recorded of 0.035 seahorse m^{-2}. This high density found could be responsible for some migration amongst the adult population to cope with competition for food and holdfasts. Apart from mortality, this

could explain the fact that some animals were only sighted once throughout the entire survey period.

In this study, the preliminary experiment proved that photo-identification is an efficient methodology to successfully identify juvenile seahorses ageing from 6 month old. In younger individuals kept in captivity, spot patterns are already present as it starts to manifest at 3 month of age (Jorge Palma, pers. obs.). Nevertheless, the spots are still too small and difficult to distinguish. In order to accurately assess the spot pattern at this age, a longer period of handling would be necessary and thus increasing the stress related to this methodology in a very sensitive life period. In addition, the reported migration of juveniles before settlement can bias the assumptions for abundance estimates that require that catchablity is constant for all animals (marked and unmarked) for all sampling periods. Hence, the abundance for each site was estimated considering only the adult population.

In conclusion, photo-identification is a useful tool for mark-recapture studies and can be successfully used to survey population abundance of *H. guttulatus* in consecutive years. This tool relies on a free open source software rendering it extremely low cost when compared to other methods in use. This could be of great value when considering the assessment of seahorse populations that are dwindling or at low densities and monitoring population fluctuations.**Acknowledgments:** Miguel Correia was supported by a PhD grant (FCT - Portugal) (BD/41020/ 2007). The study was supported by the scientific projects INAQUA and HIPPOSAFE (Fundação para a Ciência e Tecnologia, ref. PTDC/MAR/122616/2010). Thanks are also due to Project Seahorse (http://seahorse.fisheries.ubc.ca/) for providing logistic support for underwater surveys and to all the volunteers that have participated in the data collection. The manuscript benefited from the comments of two anonymous referees.

Chapter 6: Can artificial holdfast units work as a habitat restoration tool for long-snouted seahorse (*Hippocampus guttulatus* Cuvier)?

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6.1 Abstract

The recent decline of the seahorse populations in the Ria Formosa lagoon could indicate the presence of a stressful factor due to habitat loss. Artificial structures have been successfully used as a recovery tool to cope with habitat degradation in many countries but none for this seahorse species (*Hippocampus guttulatus*). Four different artificial holdfasts (S1-S4) were tested in laboratory for seahorse preference under different conditions and different holdfast densities. Seahorses, both juveniles and adults, preferred the holdfast S4, consisting of a "*Codium*-like" polyethylene nautical rope, even when submitted to different water flows. Preferred holdfast density was 156 holdfast.m^{-2}, and most of seahorses were observed grasping at the base of these structures (0-10cm in height). This study provides preliminary data and promising results on an approach to designing artificial holdfasts for seahorses in low complexity damaged or depleted areas. The use of these structures may contribute to the settlement of seahorse populations, thus broadening their potential habitat as part of a wider restoration strategy.

Keywords: Habitat enrichment; Artificial structures; *Hippocampus guttulatus*

6.2 Introduction

Typically, seahorses have a sparse distribution, low mobility, small home ranges, low fecundity, lengthy parental care and mate fidelity, rendering them vulnerable to overfishing and habitat damage (Foster & Vincent, 2004). Most seahorse species use their prehensile tail as a means to grasp different holdfasts, from sponges to coral, seagrass, mangrove branches and even artificial structures (Foster & Vincent, 2004; Harasti *et al.*, 2010; Hellyer *et al.*, 2011). Although some seahorse species prefer a particular holdfast

type (Rosa *et al.*, 2007), others like *Hippocampus guttulatus* exhibit no obvious preference although occurring in seagrass dominated habitat (Curtis & Vincent, 2005).

The long-snouted seahorse, *H. guttulatus*, is a European species, which occurs in the Ria Formosa lagoon, South Portugal, along with the short-snouted seahorse *H. hippocampus*. The greatest population size recorded for this species, throughout its range, was recorded in the early 2000's (Curtis & Vincent, 2005), however recent field data showed a significant decrease in seahorse populations within this lagoon (94% and 73% for *H. guttulatus* and *H. hippocampus* respectively) (Caldwell & Vincent, 2012). Although the causes for such declines remain unknown, human related activities (fisheries, including illegal fishing, anchoring and dredging) and natural changes in the Ria's dynamics (e.g. silting events and shifting currents), may be the main causes for an overall habitat loss (Curtis *et al.*, 2007). In the Ria Formosa, some natural *Zostera noltii* beds have been replaced by clam farms (*Guimarães et al.*, 2012), harbors, industries and coastal constructions, or dredged to open and maintain navigation channels, such as the opening of a new inlet in Fuseta island and channel dredging that are destroying vast areas of this species (Cunha *et al.*, 2014). These anthropogenic activities and natural events are known to alter the seahorses' habitat conditions and reduce the amount of natural holdfast available, essential for seahorse settlement (2010, Correia pers. obs.). Although *H. guttulatus* is not exclusively found in seagrass beds, this type of habitat is one of the most important as for feeding and protection of this species (Curtis & Vincent, 2005). Considering that the lack of holdfast availability may explain the disappearance of some populations within the Ria (Curtis *et al.*, 2007), the use of artificial holdfasts could help increase of the habitat complexity that would encourage seahorse population settlement and potentially reduce the declines.

Increased habitat complexity has been recognized to have a positive influence on the diversity and abundance of marine organisms (Silvertown, 2004). Structural complexity can be provided by plants in ecosystems by density and form (Silvertown, 2004). Diversity and abundance of marine species is usually greater in seagrass beds than in non-vegetated habitats (Hutchings *et al.*, 1991; Kirkman *et al.*, 1991; Orth, 1992; Edgar *et al.*, 1994; Lee *et al.*, 2001), as seagrass increases the amount of physical structures usable as living space, the number of microhabitats, promotes the sediment deposition and stabilization, acts as a food resource and provides protection from predators (Lee *et al.*, 2001). Many species of the Syngnathidae family, which include seahorses, pipefish and

seadragons, have been found in higher densities inside seagrass beds rather than in non-vegetated areas (Teixeira & Musick, 1995; Diaz-Ruiz *et al.*, 2000; Bell *et al.*, 2003; Kendrick & Hyndes, 2005). Seagrasses are globally threatened marine habitats (Shepherd *et al.*, 1989; Short & Wyllie-Echeverria, 1996; Spalding *et al.*, 2003) that have been degraded primarily by human activities (Lee *et al.*, 2001; Bell *et al.*, 2002; Shahbudin *et al.*, 2011) with a marked decline along some European coasts; i.e., *Zostera marina* in the Wadden Sea (Wolff, 2000) and *Posidonia oceanica* in the Corsican coast (Pasqualini *et al.*, 1998; 1999), with previous areas of 8000km^2 and 1400km of seagrass beds, respectively.

In many countries, artificial seagrass has been used as a method to replace the damaged natural seagrass ecosystem providing a marine habitat for various marine organisms, nursery ground for juveniles, habitat and protection for small fishes (Sogard, 1989; Sogard & Able, 1994; Kenyon *et al.*, 1999; Lee *et al.*, 2001; Shahbudin *et al.*, 2011). Different materials have been used to build these structures, from polypropylene/polyethylene and nylon ribbons to Dorken Advance Engineer Rubber (Lee *et al.*, 2001; Sirota & Hovel, 2006; Fernandez *et al.*, 2009; Hellyer *et al.*, 2011; Shahbudin *et al.*, 2011).

This study tested the holdfast preference for *H. guttulatus*, under controlled conditions, comparing different artificial structures that mimic the most important recorded natural holdfasts for this species, such as *Codium* spp. and seagrass (*Zostera noltii*, *Zostera marina* and *Cymodocea nodosa*). The results from this experiment will be used to inform guidelines for trialing artificial holdfast units in focal areas within the Ria Formosa lagoon as a mechanism to help the recovery of declining seahorse populations.

6.3 Materials and Methods

6.3.1 Artificial Holdfast Units

Four different holdfast types were tested and assembled in a common metal structure coated with plastic, or Artificial Holdfast Unit (AHU) (Figure 1). Each AHU measured 40x40cm with a 10x10cm grid. The different holdfast materials tested were i) 0.5cm "seagrass-like" black polyethylene plastic strips (S1); ii) 0.5cm Ø polyethylene nautical green rope (S2); iii) 0.6cm Ø "*Codium*-like" rigid plastic strings (S3); iv) 1.6cm Ø

"*Codium*-like" polyethylene nautical rope (S4). Each holdfast type measured 40cm long and was marked every 10cm with a black cable tie. These markings were used to be able to record the preferential location of seahorses in each holdfast.

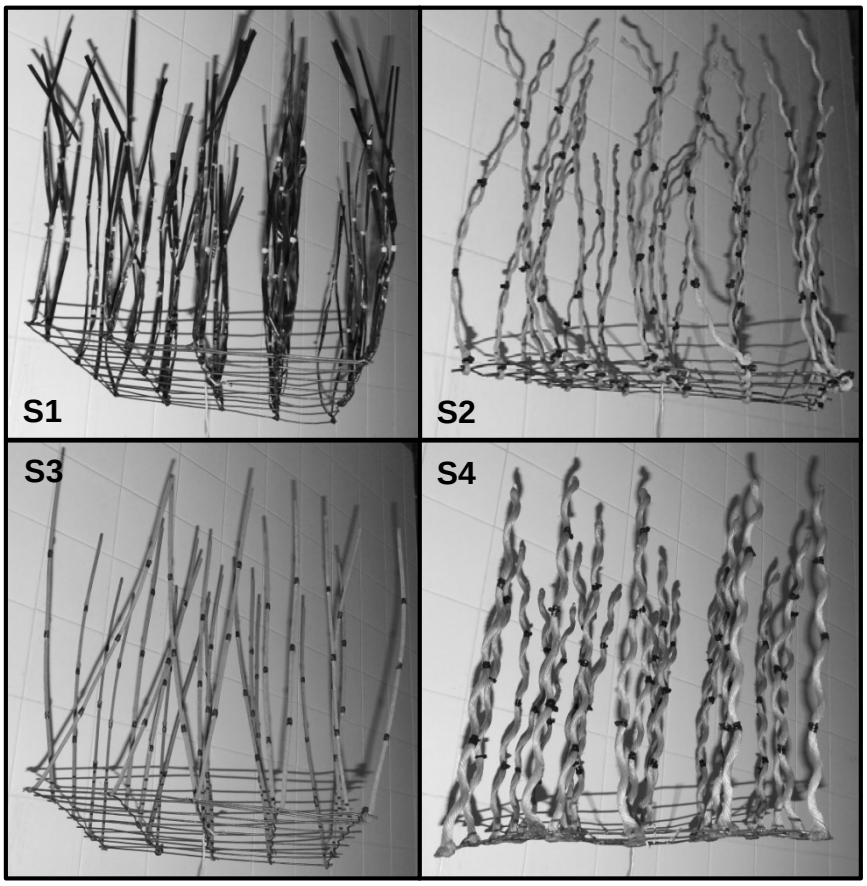

Figure 6.1: Different holdfast types used to assess seahorse preference: S1 - 0.5cm "seagrass-like" black plastic strips (S1); S2 - 0.5cm Ø nautical green rope, S3 - 0.6cm Ø "Codium-like" rigid plastic strings (S3) and S4 - 1.6cm Ø "Codium-like" bendable nautical rope.

6.3.2 Experimental design

Seahorses were placed and maintained in an 1800 litre fiberglass raceway tank (4.5m x 1m x 0.4m), assembled in an open flow-through system. The tank was divided by a polyethylene mesh panel (1cm Ø) in 3 equal sections (latter used as replicates) measuring 1.5 m x 1 m x 0.4 m. Light, water-flow and aeration were constant throughout the experiment and identical in each section. Water was filtered through an UV light with a temperature and salinity averaging 16.5 ± 0.5 ºC and 36 ± 1‰, respectively. Water quality parameters were monitored every 3 days and were stable throughout the experiment, with

ammonia values always below detectable levels, nitrate <0.3mg L^{-1} and nitrite <12.5 mg L^{-1}. In each experiment, a total of 30 adult (15 males and 15 females) captive bred seahorses, *H. guttulatus* (F_2) were used and randomly distributed in each of the three replicates in a 1:1 gender ratio (5 males and 5 females per replicate). Each seahorse was tagged with a soft braided fishing line necklace with a specific bead color for individual recognition. After the termination of the observation period, the adults were removed from the tank and a new experiment was done with 30 juvenile seahorses (10 per replicate) using the same methodology as for the adults. Juveniles used in this experiment came from the same brood and aged 4 months old with an average length of 12 ± 1.4cm. This size class was selected as it is the most common size class of juveniles observed in the wild in the Ria Formosa. Juveniles were not divided by gender as individual sex was not distinguishable. Juveniles were then tested for holdfast preference using the same methodology as for adult seahorses.

All seahorses were sampled at the start and at the end of the experiment and the weight and height were recorded. In order to minimize handling and stress during sampling, as an alternative to the measuring protocol proposed by Lourie *et al.* (1999a), a simplified protocol was used. Instead of the three measurements proposed by Lourie et al. (1999) (the sum of head, trunk and tail lengths) seahorses were measured by the sum of the head length and total height (from the top of the coronet until the tip of the tail). Seahorse averaged 17.9 ± 3.1cm and 17.1 ± 0.9g; and 9.5 ± 0.4cm and 2.4 ± 0.3g for adult and juveniles, respectively. There were no significant differences in seahorse length or wet weight between treatments, within or between sexes, or replicates at the start of the experiment (ANOVA, $p>0.05$).

During the 2 days acclimation period and throughout the experiments, adult and juvenile seahorses were fed *ad libitum* with a mix of frozen shrimp (*Palaemontes varians*) and live mysids (*Mesopodopsis slabberi*). After the beginning of the experiments, seahorses were only fed *ad libitum* once a day, after the observation period, to avoid feeding effects on seahorse behavior, as most feeding activity was observed during the first hour after the feed was provided. Each replicate tank was siphoned on a daily basis, after the observation period and prior to feeding to remove uneaten feed, feces and other detritus. At the end of the experiments, the necklace tags were removed and seahorses were returned to their grow-out tanks. No injuries, disease outbreaks or mortalities were recorded during the course of this study.

6.3.3 Holdfast preference

6.3.3.1 Holdfast type and location

One of each holdfast type (S1 to S4) was placed in each replicate section, equidistant (20cm) from each other. During the 2 days acclimation period, 10 seahorses were released into each replicate tank and allowed to freely choose the holdfast to grasp. Location, individual identification, gender and holdfast preference were recorded for each individual on a 60 min interval, 6 hours per day (from 09:00-12:00 and 14:00-17:00) for 10 days, completing a total of 800 single observations per replicate.

6.3.3.2 Holdfast density

Considering the results obtained in the previous experiment addressing the holdfast type preference, the holdfast type used in this experiment (S4) was the one that yielded the best results in the previous experiment. In order to assess seahorse holdfast density preference, 3 AHU of different densities were used per 3 replicates. Holdfast density was 9, 25 and 41 holdfasts per AHU (56, 156 and 256 holdfast.m^{-2}). Again, preference for holdfast density for adults and juveniles was recorded every 60 min, 6 hours per day (from 09:00-12:00 and 14:00-17:00) during 10 days, completing a total of 800 single observations per replicate.

6.3.3.3 Hydrodynamic effect

6.3.3.3.1 Holdfast type

In order to test the effect of water current on holdfast preference, two power pumps Eheim®159GPH (600L.h^{-1} max. capacity) were placed in the tank. Prior to the introduction of seahorses in the experiment tank, current flow was measured and adjusted to 1 m.s^{-1} and directed at the AHU. This value is equivalent to the average maximum current flow recorded in the Ria Formosa lagoon (Pacheco *et al.*, 2010). A pairwise comparison between holdfast types (S1 to S4) was conducted. Each day, a combination of 2 different holdfast types was tested during 2h30min and preference recorded in a 15 min interval. At the beginning of each observational period, 10 seahorses were placed in the tank while the pumps were working to assess immediate holdfast preference, as the water flow used in this experiment is greater than the long term swimming capability of seahorses, as observed in the wild and in laboratory. As the water flow used in this experiment mimic the maximum water flow occurring under natural conditions, this experiment allowed to observe the seahorse holdfast preference behavior when exposed

to more severe hydrodynamic conditions. The duration of this experiment aimed to simulate the tidal peaks that naturally occur in the wild. This experiment lasted for three consecutive days (one replicate per day), and each replicate was tested for preference with an overall 330 single observations per combination.

6.3.3.3.2 Holdfast density

Two AHU's (S4) of the 2 different densities that yielded the best results in the previous experiment (section 3.2 - 156 and 256 holdfast.m^{-2}) were placed in the tank, equally distant to the water pumps. Tank setup, data collection and experimental period was the same as described above (section 3.3.1)

6.3.3.3.3 Water flow

This experiment aimed to test the effect of current on the ability of a seahorse to grasp a holdfast. Seahorses were placed in a separate section of the raceway prior to the beginning of the experiment. Considering the results obtained in section 3.1 and 3.2, three AHU of the preferred holdfast type (S4) and density (156 holdfast.m^{-2}) were used and placed inside the experimental tank, parallel to the water pump. Each AHU was placed in parallel and equally distant to each other, at the same time that a submersible water pump Eheim® 159GPH (600L.h^{-1} max. capacity) created a water flow of 1, 0.3 and 0.1m.s^{-1}, respectively, when passing through the structures. Before the start of the experiment, seahorses were moved to the main experiment section, with the water flow and left to freely choose an AHU. Observations were made twice a day for 2h30m each (10:00-12:30 and 14:00-17:30), in a 15 min interval. The experiment lasted 2 days completing 440 single observations.

6.3.4 Statistical analysis

Seahorse holdfast type preference, density and water current were compared using a one-way ANOVA. Tukey's post-hoc test was used to identify whether there were differences in preference within each replicate group. Gender preference was tested using a two-way ANOVA. In all test procedures, data was analyzed for normality and homogeneity, and whenever one of these requisites was not present, alternative non-parametric tests were used (Zar, 1999). All statistical analysis was performed for a significance level of 0.05, using Statistica 6.0 software (StatSoft Inc. Data).

6.4 Results

6.4.1 Holdfast preference

6.4.1.1 Holdfast type and location

Using one-way ANOVA, both juvenile and adult seahorses showed a significant preference to grasp to holdfast S4 (Figure 2), compared to the other three holdfast types ($F=35.29$, $P<0.001$; $F=111.6$, $P<0.001$ for juveniles and adults, respectively). No statistical difference was observed in preference between genders ($F=0.21$, $P=0.653$) with both males and females preferring the S4 holdfast.

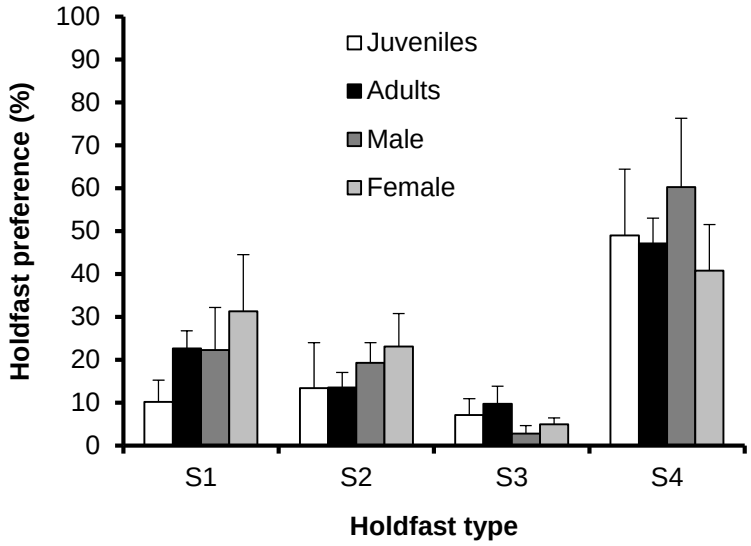

Figure 6.2: Holdfast preference (%) for juveniles and adult seahorses comparing four different holdfast types (S1-S4). Preference by sex is detailed for adult seahorses. Vertical bars represent standard deviation.

Regardless of the holdfast type, seahorses were mostly located near the base of the holdfast (0-10cm; $P<0.05$), and only occasionally on higher sections (>10cm; Figure 3). During the course of this experiment, courtship behaviour was observed and no mortality occurred.

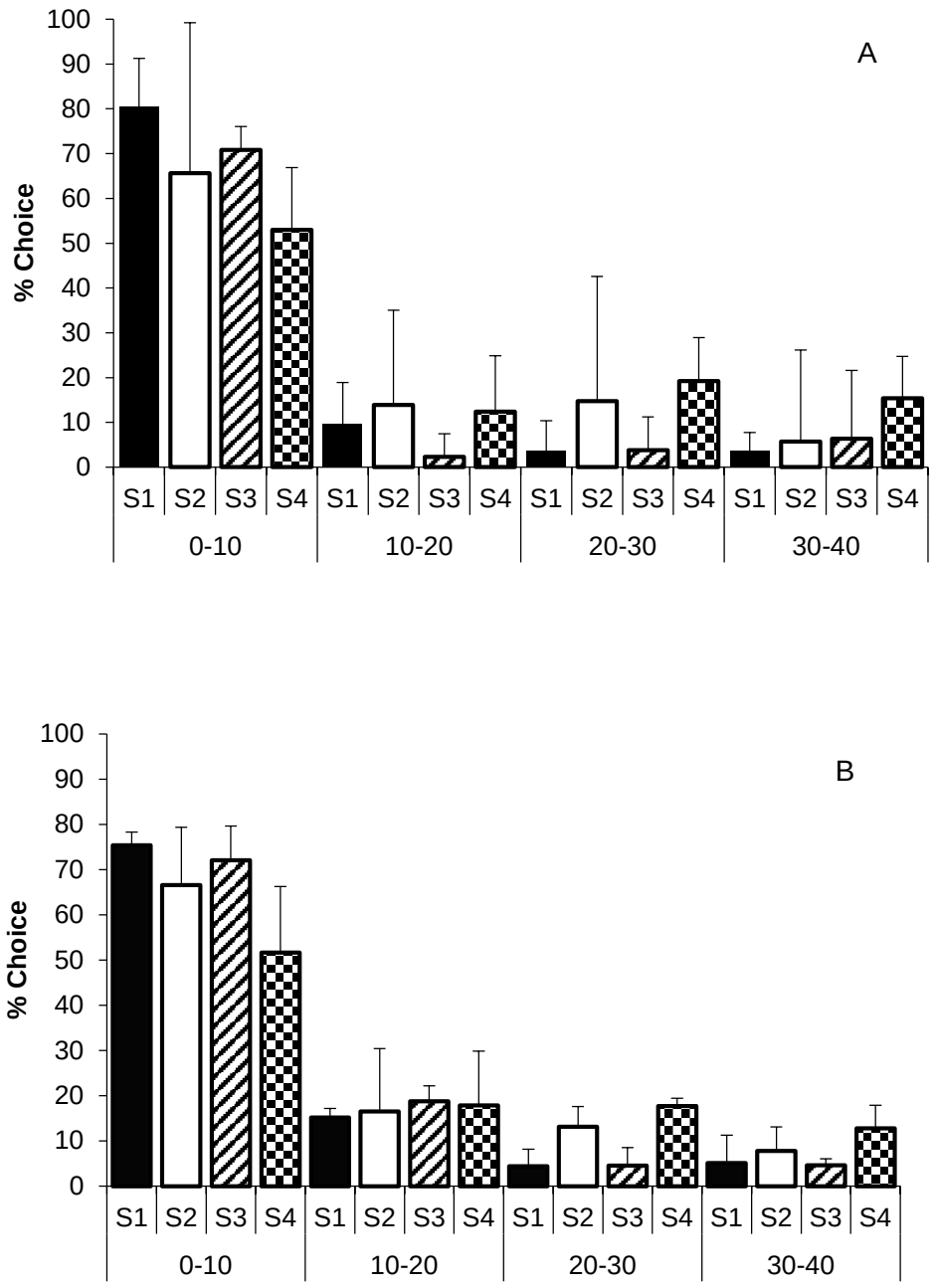

Figure 6.3: Location preference (cm) for *H. guttulatus* adults (A) and juveniles (B) in each holdfast type tested (S1-S4). Vertical bars represent standard deviation.

Even when submitted to a current effect, both juvenile and adult *H. guttulatus* showed a preference for S4 holdfast. Significant differences were found, using one-way ANOVA, for both groups tested ($F=14.05$, $P<0.001$; $F=15.39$, $P<0.001$, respectively). Preference for S4 holdfast was significant higher when compared with all other holdfasts tested for both juvenile and adult seahorses (Table I).

Table 6.I: Holdfast preference (%) for different holdfast types (S1-S4), at a water flow of 1 $m.s^{-1}$, for juvenile and adult *H. guttulatus*. Tukey Multiple comparison results are shown in each section. Significant differences are indicated with *.

		Holdfast type			
		S1	**S2**	**S3**	**S4**
Juveniles		18.5 ± 7.6%	22.9 ± 8.5%	18.9 ±8.6%	39.7 ± 7.3%
Tukey	**S1**	---	1.672	0.157	7.953*
	S2	1.672	---	1.515	6.282*
	S3	0.1 57	1.515	---	7.796*
	S4	7.953*	6.282*	7.796*	---
Adults		18.7 ± 13.9%	16.7 ± 13.7%	17.8 ± 8.6%	46.8 ± 6.0%
Tukey	**S1**	---	0.533	0.214	7.585*
	S2	0.533	---	0.319	8.117*
	S3	0.214	0.319	---	7.799*
	S4	7.585*	8.117*	7.799*	---

6.4.1.2 Holdfast density

In this experiment the S4 holdfast type was used, as seahorses had shown the strongest preference for it in the previous experiment. Statistical differences were found, using one-way ANOVA, in holdfast density preferences ($F=119.90$, $P<0.001$; $F=94.46$, $P<0.001$, for juveniles and adults, respectively). Both juvenile and adult *H. guttulatus* showed preference for medium and high holdfast densities when compared to the lowest density (Figure 4). Using Tukey's multiple comparison test, no significant differences were found between medium and high densities ($q=0.961$, $P>0.05$; $q= 2.983$, $P>0.05$, for juveniles and adults, respectively), while significant differences were found between low density and the latter (low *vs* medium: $q=17.290$, $P<0.05$; $q=17.300$, $P<0.05$ for juveniles and adults, respectively; low *vs* high: $q=16.330$, $P<0.05$; $q=20.280$, $P<0.05$ for juveniles and adults, respectively).

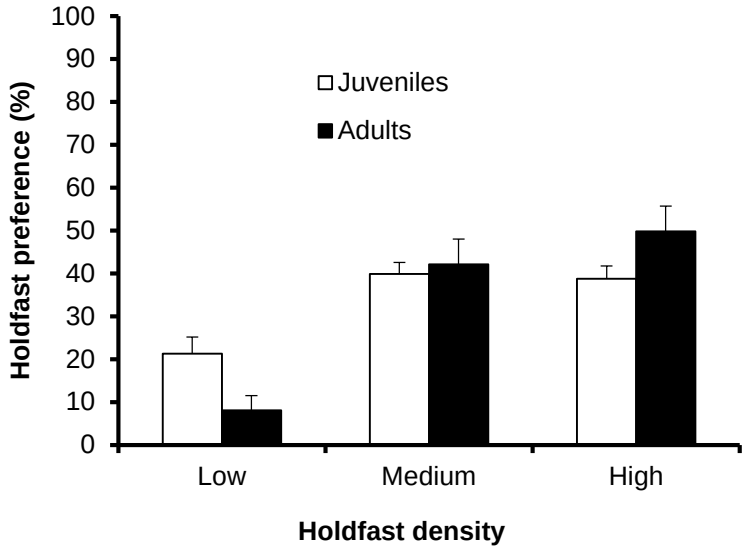

Figure 6.4: Preference (%) for holdfast S4 densities (56, 156 and 256 holdfast.m^{-2}), for juvenile and adult *H. guttulatus*. Vertical bars represent standard deviation.

When submitted to a current effect, both juvenile and adult *H. guttulatus* showed a preference for S4 holdfast at medium density (156 holdfast.m^{-2}) with occupancy of 69.4±14.1% for adults and 63.7±6.9% for juveniles. Significant differences were found for both groups tested ($P<0.001$).

6.4.1.3 Water flow

Juveniles showed significantly lower tolerance to stronger currents, whereas adults resisted to higher currents. Juveniles preferred grasping to the S4 holdfast (46.9±17.4%) at lowest water current, whereas adults preferred stronger water flows (31.8±16.3% and 39.7±15.7% for 1 m.s^{-1} vs 0.1 m.s^{-1}, respectively) (Figure 5). Statistical differences (KW test) in both experiments ($P<0.05$) and Dunn's multiple comparison test showed significant differences ($P<0.05$) between 1 m.s^{-1} *vs* 0.1 m.s^{-1} for juveniles and 0.3 m.s^{-1} *vs* 0.1 m.s^{-1} for adults.

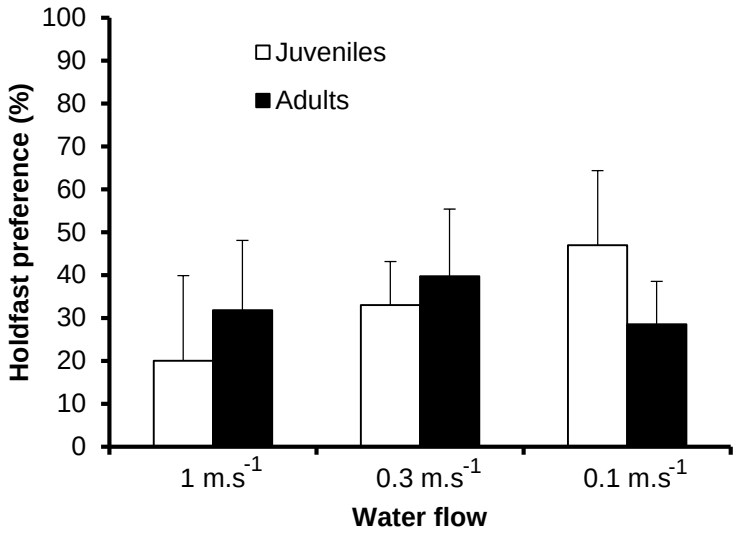

Figure 6.5: Holdfast preference (%) under different water flows, using S4 holdfast at 156 holdfast.m^{-2}, for juvenile and adult *H. guttulatus*. Vertical bars represent standard deviation.

6.5 Discussion

H. guttulatus is the most abundant seahorse species in the Ria Formosa and, when compared to the sympatric species (*H. hippocampus*), it has shown a more drastic reduction in numbers in the past 10 years (94% and 73%, respectively) (Caldwell & Vincent 2012). As *H. guttulatus* favors more complex habitats than *H. hippocampus*, and were naturally found using both biological and artificial holdfasts (Curtis & Vincent 2005), this species was used to test the potential of artificial holdfast units (AHUs) in this study. The AHUs designed for this study were based on the natural preferences recorded for this species. As *H. guttulatus* was associated with seagrass beds (Curtis & Vincent, 2005), the S1 holdfast type was designed to simulate the seagrass leaves as they were of the same shape, size and thickness. Curtis & Vincent (2005) reported that seahorses were found on seagrass beds (58.6%) and on macroalgae (20.8%) considering the total covered area. In addition, and according to our own observations in the wild, this species has been observed grasping on macro-algae, particularly *Codium* spp. Therefore, the S2 to S4 holdfasts aimed to mimic different thickness and hardness of *Codium*-like holdfasts, each providing different structural behavior when placed in a hydrodynamic environment. S3 holdfasts were tested as an artificial structure that would provide stiffer holdfasts that could be potentially suitable for seahorse holdfast, considering the Ria Formosa's hydrodynamics. Although it was expected that *H. guttulatus* would prefer the S1 holdfast, as it mimics the seagrass bed, this study showed this species showed significant

preference for the S4 holdfast type. Teske *et al.* (2007) reported that *Codium* spp. was the preferred holdfast for *Hippocampus capensis*, compared to all other available holdfasts, even the more abundant *Zostera* sp. These authors also suggested that the fact that more seahorses were found grasping seagrass might be due to its higher availability and not because it was the preferred holdfast. In addition, when comparing the four different holdfast types in strong current situation (1 $m.s^{-1}$), there was again a clear preference for the S4 holdfast type. This may be due to the fact that the S4 holdfast provided a more stable structure for seahorses, which enables them to camouflage and hunt for prey more effectively, at the same time it grants them protection against stronger currents. This will probably allow broadening their habitat, allowing the *H. guttulatus* population settlement in areas that no longer have conditions for seagrass beds or other natural structure to exist. Similarly, Clynick (2008) reported that the use of swimming pool nets granted a good habitat for seahorses in Sydney Harbour, a highly human impacted area. The fact that seahorses were found grasping all the tested holdfast, emphasizes the holdfast versatility reported for *H. guttulatus* in the Ria Formosa, as they grasp different holdfasts like seagrass blades, macroalgae, tunicates, bryozoans, polychaete tubes, sea urchins, and artificial structures (Curtis & Vincent, 2005). There were no individual or sex related differences between holdfast preferences, demonstrating a clear preference for one particular AHU type from this study (holdfast S4).

As for holdfast density, seahorses showed preference for densities of 156 and 256 $holdfasts.m^{-2}$ (S4 holdfast type). Seahorses have been found in seagrass beds (*C. nodosa*) with a mean leaf height ranging from 11.1 to 34.2 cm and mean shoot density ranging from 233.3 to 848.3 shoots m^{-2} (Curtis & Vincent, 2005), which support our results. Higher densities were not tested as no significant differences were found between both medium and high densities (156 and 256 $holdfasts.m^{-2}$). Prey density usually increases with increasing habitat complexity, resulting in higher predator feeding success at intermediate complexities (Crowder & Cooper, 1982; Canion & Heck, 2009). According to James & Heck (1994), even in high structure densities (up to 3,032 $seagrass\ blades.m^{-2}$) predation success is not significantly affected by sedentary predation, in contrast with active predation. Curtis & Vincent (2005) found that abundance of *H. guttulatus* is positively and significantly correlated with the percentage cover of vegetation and immobile benthic invertebrates, which is supported by the findings in this study.

Stressed by the highly hydrodynamic nature of the Ria Formosa lagoon, where average maximum current speed can go up to 1.25 $m.s^{-1}$ (Pacheco *et al.*, 2010) and their low mobility, *H. guttulatus* requires holdfasts in order to prevent them to be dragged away from their preferred habitats (Curtis & Vincent, 2005). In this study, as holdfast preference could be dependent on current speed and results obtained in a static environment could be misleading, the water flow experiments aimed to replicate the natural conditions thus testing the effectiveness of the different AHU's and ultimately their viability in a future deployment in focal sites. In the case of current preference, adult seahorses preferred the 0.3 $m.s^{-1}$ water flow, even when lower current was available. This might indicate the natural preference for hydrodynamic environments, which may be explained by their feeding and cryptic behavior (Foster & Vincent, 2004; Curtis & Vincent, 2005). Juveniles occupied the holdfast set at lower current speed (0.1 $m.s^{-1}$) which may be due to their inability to cope with strong water flows. This might be indicative that juveniles prefer sheltered and low current conditions in their natural habitat. Nevertheless, these assumptions require an *in situ* confirmation through underwater visual census surveys (UVC), monitoring several sites within the Ria with different hydrodynamics and thus assessing juvenile habitat preference. In fact, the need for holdfasts in a hydrodynamic environment indicate that one of the possible causes for seahorse decline in the Ria Formosa reported by Caldwell & Vincent (2012) could be in fact be due to habitat loss or degradation. Therefore, it seems to be important to continuously monitor habitat changes and seahorse population trends during an extended period of time.

Seagrass destruction within the Ria Formosa and in other North Atlantic Ocean or Mediterranean Sea locations (Cunha *et al.*, 2005) could result in a reduction of areas of *H. guttulatus* habitat, and hence local population sizes. Habitat degradation is one of the greatest threats to seahorse populations at low densities in a lagoon environment (Foster & Vincent, 2004; Harasti *et al.*, 2010; Vincent *et al.*, 2011). Allee effects (tendency for population to decline numerically when it gets below a certain threshold size or density) might be of important relevance in an environment with high patchiness and low population density (Vincent *et al.*, 2011). The seahorse's cryptic behavior, poor swimming ability and dependence on using the prehensile tail to grip holdfast, renders them particularly dependent upon holdfast structures for hunting and predator avoidance (Foster & Vincent, 2004; Curtis & Vincent, 2005; 2006). In many countries, artificial seagrass has been recognized as an alternative method to cope with dwindling natural

seagrass ecosystems and is becoming widely used as an alternative marine habitat for various marine organisms (Sogard & Able, 1994; Kenyon *et al.*, 1999; Lee *et al.*, 2001; Shahbudin *et al.*, 2011). Artificial seagrass units (ASUs) have been used to investigate whether seagrass density (Bell *et al.*, 1987) or distance from natural seagrass (Sogard, 1989) affected settling fish and decapods and found that, although artificial seagrass has lower faunal assemblage capability when comparing to natural seagrass, they have proven to be a valid replacement, even if temporarily while the natural habitat recovers. Similarly, ASUs have been used to quantify spatial settlement patterns in enclosed embayment (Jenkins *et al.*, 1996; Jenkins & Sutherland, 1997). These structures have also been shown to provide a suitable habitat for small fishes, increase prey density, promote nursery grounds for juveniles, and predatory protection for small fishes, thus playing a useful role in maintaining balance in marine environments when the natural habitat has been degraded or destroyed (Shahbudin *et al.*, 2011).

The use of artificial structures to rehabilitate damaged areas including coral reefs, saltmarshes or other coastal areas, is still a subject of debate (Hauser *et al.*, 2006; Sirota & Hovel, 2006; Fernandez *et al.*, 2009; Vega Fernández *et al.*, 2009; Moberg *et al.*, 2011). Artificial structures have been referred to as tools for fish population reestablishment as they provide shelter and feeding grounds for many communities (Ambrose & Anderson, 1990; Bohnsack *et al.*, 1994; Charbonnel *et al.*, 2002; Zalmon *et al.*, 2002; Claudet & Pelletier, 2004). Nevertheless, they may just act as an aggregation device for fish rather than increasing overall abundance (Grossman *et al.*, 1997; Pickering & Whitmarsh, 1997). Although this argument may be true for more mobile species (Santos & Monteiro, 1997; Charbonnel *et al.*, 2002), the use of these structures has the potential to provide a long term beneficial effect on more sedentary species like seahorses.

Like other seahorse species, *H. guttulatus* have low mobility and a small home range (Foster & Vincent, 2004; Curtis & Vincent, 2005; Caldwell *et al.*, 2011). These characteristics render the seahorses vulnerable to human and natural disturbances which ultimately may be responsible to habitat loss or degradation (Curtis *et al.*, 2007). In fact, habitat degradation may limit immigration and diminish the re-pairing of eventual widowers or of a disrupted couple (Vincent & Sadler, 1995; Foster & Vincent, 2004; Vincent *et al.*, 2005). Habitat patchiness will result in a sparse distribution of seahorses and therefore may decrease the opportunities for sexual interaction and so contributing to a significant long term population decrease. The use of these artificial structures may

provide both seahorse species (*H. guttulatus* and *H. hippocampus*) an improved habitat, promoting the populations settlement and ultimately maximizing both individual and sexual interaction. This study provided the first step in establishing how AHUs might be used as a management tool for enhancing declining seahorse populations in the Ria Formosa lagoon.

This study provides preliminary data and promising results on an approach to designing artificial holdfasts for seahorses in low complexity damaged or depleted areas. The use of these structures may contribute to the settlement of seahorse populations, thus broadening their potential habitat as part of a wider restoration strategy. In order to assess the viability of the use of these structures as habitat enrichment for local seahorse populations, more in depth *in situ* trials will be required with surveys of seahorse populations before and after deployment.

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Chapter 7: Effects of artificial holdfast units on seahorse density in the Ria Formosa lagoon, Portugal.

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7.1 Abstract

A significant decline in the seahorse populations in the Ria Formosa has been recently reported and holdfast availability suggested as a particularly important variable that influences the abundance of the long-snouted seahorse (*Hippocampus guttulatus*) and the short-snouted seahorse (*H. hippocampus*). In order to test the usefulness of artificial holdfast units (AHU) to enhance habitat recovery, several of these structures were deployed at four different locations with distinct environmental characteristics and surveyed for seahorse abundance during a 6 months period. All AHUs were colonized by seahorses within a month after deployment, reaching a maximum density of 13.1 seahorse m^{-2} at one of the sites. Results suggest that these AHUs have the potential to aggregate seahorses in damaged habitats but have limited effect when placed close to natural high complexity habitats. The results from this experiment provide useful guidance in the use of artificial structures to improve degraded seahorse habitats in other similar situations, as part of management plans for seahorse population recovery.

7.2 Introduction

Worldwide, seahorse populations are threatened mostly due to degradation of their habitats, incidental capture in fishing gear (by-catch), and over-exploitation for use in the aquarium trade, curiosities and traditional medicines (Vincent, 1996). Seahorses are particularly vulnerable to population decline because of their distinctive life history, behavior and ecology: they provide lengthy and vital parental care for small broods, exhibit low mobility and site-fidelity (Foster & Vincent, 2004).

In addition, seahorses inhabit shallow, coastal areas worldwide, where anthropogenic disturbances tend to be most frequent and severe (Bell *et al.*, 2003). Due to their peculiar

morphology, seahorse use their prehensile tail to grasp different holdfasts, from sponges to coral, seagrass, mangroves and even artificial structures (Foster & Vincent, 2004; Harasti *et al.*, 2010; Hellyer *et al.*, 2011) as their life-history is fully dependent of this behavior. Although some seahorse species prefer particular holdfast types (Rosa *et al.*, 2007), others like *Hippocampus guttulatus* exhibit no obvious preference, grasping both natural and artificial structures (Curtis & Vincent, 2005).

The long-snouted seahorse (*H. guttulatus*) and the short-snouted seahorse (*H. hippocampus*) are sympatric species and the only two that live in the Northeast Atlantic (Pardo *et al.*, 2007), including the Ria Formosa lagoon, South Portugal. Although the highest population abundances of these species have been recorded in the Ria Formosa in the early 2000's (Curtis & Vincent, 2005), recent findings by Caldwell & Vincent (2012) showed a significant decrease in those populations within this lagoon: 94% for *H. guttulatus* and 73% for *H. hippocampus*, respectively. The causes for such decline are currently under investigation (Chapter 2), but human related activities (e.g. fisheries, including illegal fishing, anchoring/mooring and dredging) and natural changes in the Ria's dynamics (e.g. silting events and shifting currents), seem to be the main causes for an overall habitat loss (Curtis *et al.*, 2007). The Ria Formosa sustains numerous human related activities including clam farming (Guimarães *et al.*, 2012), harbours, shipyards, coastal constructions and episodic dredging activity (to open and maintain navigation channels), which combined are responsible for the destruction of vast areas of potential seahorse habitat (Cunha *et al.*, 2013). The combination of these anthropogenic activities and natural events are known to alter the seahorses' habitat conditions and reduce the amount of natural holdfasts available, essential for seahorse settlement (2010, Correia pers. obs.). Furthermore, there are potential climate change effects due to a consistent documented increase in the average water temperature in the lagoon and its associated effects (e.g. dissolved oxygen), a factor that is also known to have a negative impact on seagrass beds (Cunha *et al.*, 2013), thus reducing holdfast availability. Therefore, as the lack of holdfast availability may explain the disappearance and/or desegregation of some populations within the Ria (Curtis *et al.*, 2007), the use of artificial holdfasts is a potential tool to increase habitat complexity thus encouraging seahorse population settlement and mitigating some of the causes of their decline.

Increased habitat complexity has been recognized as beneficial to the biodiversity and abundance of marine organisms (Silvertown, 2004). In many countries, artificial

structures have been used as a method to replace damaged natural ecosystems providing a habitat for various marine organisms, nursery grounds for juveniles, and habitat and protection for small fishes (Sogard, 1989; Sogard & Able, 1994; Kenyon *et al.*, 1999; Lee *et al.*, 2001; Shahbudin *et al.*, 2011). Different materials have been used to build these structures, from polypropylene/polyethylene (Sirota & Hovel, 2006; Fernandez *et al.*, 2009; Hellyer *et al.*, 2011; Correia *et al.*, 2013) and nylon ribbons (Lee *et al.*, 2001) to Dorken Advance Engineer Rubber (Shahbudin *et al.*, 2011).

This study aimed to test the effect of using artificial holdfast units (AHUs) under natural conditions on habitat use and density of seahorses in the Ria Formosa lagoon.

7.3 Materials and Methods

7.3.1 Site description

This study was conducted at eight locations (Figure 1) in the Ria Formosa lagoon, South Portugal (36º59´N, 7º51´W). Four of these locations were chosen for AHU deployment as they were previously surveyed using underwater visual census (UVC) methodology (Chapter 3) and their low seahorse abundance (<0.02 seahorse m^{-2}) and limited holdfast coverage was confirmed. In close vicinity to each of these locations, four other locations were chosen to be surveyed as control locations. Caldwell & Vincent (2013) referred a maximum of 150 m movement for *H. guttulatus* in the Ria Formosa. So, under this premise, each of the four AHU deployment sites and their respective control sites were deployed over 200 meters from each other to prevent home range overlap. Three of these control locations were the same that were previously surveyed in Chapter 4. Each selected site had comparable characteristics regarding habitat complexity, depth, hydrodynamics and human impact (Table I), whereas Site 3 had no seahorse populations in a 100 m^2 radius. Habitat complexity was considered as low if bottom coverage, i.e. number of holdfasts available, was under two holdfasts m^{-2}; medium for 2 to 10 holdfasts m^{-2}; and high for more than 10 holdfasts m^{-2}. Holdfast distribution was considered as patchy when there was a distance between holdfasts in more than a 3-meter radius. At each sampling, human related activities were recorded to determine their impact in each site. Sites were considered as highly impacted by human activities when two or more activities were observed in every sampling occasion, i.e., fisheries, boat traffic and anchoring; medium

impact when at least two of those activities were observed in at least half of the sampling occasions; and low impact when less than two activities were observed in less than half of the sampling occasions.

Figure 7.1: Sites location in the Ria Formosa lagoon. AHU Prototype (P), Site 1 (1), Site 2 (2), Site 3 (3) and Site 4 (4). All AHU and Control sites were distant of more than 200m apart.

Table 7.I: Description of each surveyed site regarding depth (meters); hydrodynamics (water flow); habitat complexity in the vicinities (holdfast availability); holdfast distribution; and human impact.

Location	Depth	Hydrodynamics	Habitat complexity	Human impact
Site 1	5 - 6 m	Medium (0.5-1 $m.s^{-1}$)	High	High (fisheries and boat anchoring)
Site 2	3 - 4 m	High (0.3-0.7 $m.s^{-1}$)	Low	Medium (occasional fisheries)
Site 3	3 - 4 m	High (0.1-0.5 $m.s^{-1}$)	Low	Low (occasional human activity)
Site 4	2 - 3 m	Low (0.1-0.5 $m.s^{-1}$)	High	Low (occasional human activity)

7.3.2 Artificial Holdfast Units

The design of the Artificial Holdfast Units (AHU) used in this study was an output of a previous experiment (Correia *et al.*, 2013) where, different holdfast materials, holdfast size and density were tested under controlled conditions. Each AHU was composed by a metal grid covered with polypropylene plastic base measuring 100x100cm (with 10x10cm grid gaps) where holdfasts were attached. The holdfasts, made of 1.6cm Ø polyethylene nautical rope and measuring 40cm long were evenly distributed in the AHU, at a density of 100 holdfast.m^{-2}.

Prior to the deployment of the AHUs in the selected locations, one single prototype was placed in an area closely located to Site 2 (Table 1) in order to obtain background information before starting the experiment. This prototype, consisting of 4 AHUs, displayed in a square shape, summing a total area of 4 m^2. The prototype was deployed in early July 2012 and surveyed for 6 months on a monthly basis. In each monthly survey, the number of seahorses, species, size and overall status of the AHUs were recorded.

The prototype proved its reliability under natural conditions, so, later on, three identical replicate units (3x4 m^2), were placed 10 m apart at a same depth (Figure 2) in each of the four selected locations (Table 1). Each AHU was firmly attached to the bottom substrate with plastic covered metal pins to avoid being dragged by water currents or fishing gears. These AHUs were deployed in February 2013 and then surveyed on a monthly basis over a 6-month period (March–August 2013). Considering those four selected locations' habitat, four control sites were chosen with similar habitat characteristics, located more than 200 meters apart from the respective AHU to avoid home range overlap. The same survey methodology was used in the control areas, but with no AHU deployed. During each survey, seahorses found in the AHUs and control sites were identified for species, counted and sexed. All *H. guttulatus* found were photographed for individual identification purposes (Chapter 5). Photo-identification will determine the individual use of the AHU deployed. For accuracy and replicability, a GPS unit was used to determine the exact location of the replicate AHUs in each study area.

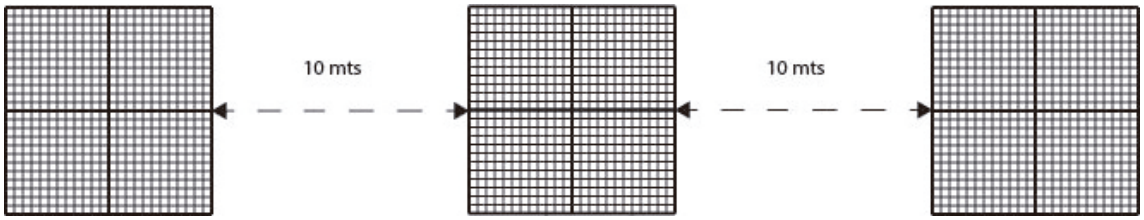

Figure 7.2: Schematic of the AHU deployed at each Site (AHU1 to 4).

7.3.3 Statistical analysis

In the prototype trial, seahorse species, seahorse density and gender differences were compared using a Student t-test. A two-way ANOVA was then used to compare seahorse abundance and gender differences between locations. Sidak's multiple comparisons test was used to identify whether there were differences in preference between each site and at different time periods (Zar, 1999).

As for the AHUs deployed in the four different locations, seahorse density was tested for statistical differences using a one-way ANOVA. In all test procedures, data were analyzed for normality and homogeneity, and whenever one of these requisites was not present, alternative non-parametric tests were used (Zar, 1999). All statistical analysis was performed for a significance level of 0.05, using Statistica 6.0 software (StatSoft Inc. Data)

7.4 Results

7.4.1 First trial - AHU Prototype

One month after deployment (July), seahorses were observed on the AHU prototype at a density of 3.5 seahorse m^{-2}. The lowest seahorse density was observed in August (2.3 seahorse m^{-2}), when the AHUs were covered by bryozoan *Zoobotryon verticillatum.* From September onwards, the bryozoan decreased its area of occupancy and completely disappeared in October 2012 again leading to a new increase in seahorse occupancy reaching a peak of abundance during December at a density of 9.5 seahorse m^{-2} (Figure 3).

Both seahorse species were observed on the AHU prototype. Maximum density of *H. hippocampus* was recorded in August (1.5 seahorse m^{-2}), while highest *H. guttulatus* density was observed in December 2012 (8.75 seahorse m^{-2}). During the six month survey period, the abundance of *H. guttulatus* was significantly higher than the *H. hippocampus* (P=0.03). No significant differences in gender were found for *H. guttulatus* and *H. hippocampus* (P=0.32 and P= 0.85, respectively).

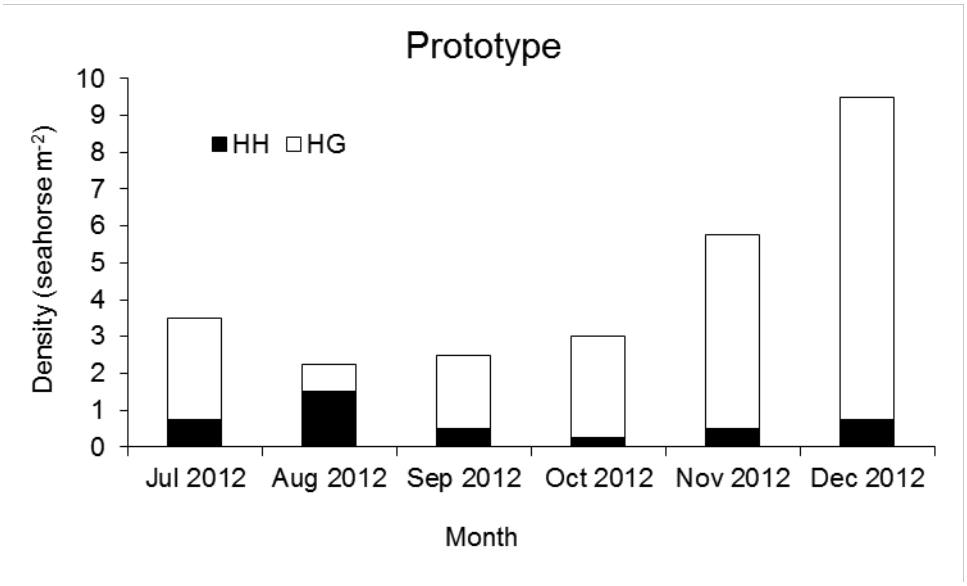

Figure 7.3: Density (seahorse m^{-2}) for *H. hippocampus* (HH) and *H. guttulatus* (HG) in the AHU Prototype at the different survey events.

7.4.2 Second trial - Artificial Holdfast Units at different locations

All AHU were occupied by seahorses one month after deployment in each of the four locations. The maximum abundance was reached in May in all sites and then a constant decrease was observed until the last survey in August (Figure 4). The highest seahorse density was observed three months after deployment in May at Site 2 (13.1 seahorse m^{-2}). In Site 4, all AHU replicates became progressively buried under sand due to continuous silting event and in July, the AHU were no longer visible so no further surveys were made in this site from that point onwards. Similar to the prototype trial, in Site 1 to 3, AHU became covered in the bryozoan *Zoobotryon verticillatum*. This was first observed 5 months after deployment in July and remained until the end of the study in August 2013.

H. guttulatus was significantly more abundant than *H. hippocampus* ($P<0.05$) in all sites and in all survey events. No gender differences ($P>0.05$) were found for both species in Site 1 and Site 4 at all survey events (Figure 5), nonetheless, in Site 2 and Site 3, significant gender differences ($P<0.05$) were found during June 2013.

Seahorse density was significantly different between Site 4 and Sites 2 and 3 ($P<0.05$) (Table II).

Table 7.II: Tukey's multiple comparison test results after comparing seahorse abundance in every site. Statistical differences are represented by (*)

	Site 1	Site 2	Site 3	Site 4
Site 1		0.18	0.70	0.18
Site 2	0.18		0.17	0.01 *
Site 3	0.70	0.17		>0.01 *
Site 4	0.18	0.01 *	>0.01 *	

7.4.3 Photo-identification

A total of 709 photographs of first and re-sighted *H. guttulatus* were shot in all sites (86 in Site AHU1, 428 in Site AHU2, 166 in Site AHU3 and 29 in Site AHU4). Recapture percentage, i.e. percentage of *H. guttulatus* re-sighted at least once in a sampling period, varied from a minimum of 5.5% in Site 1 to a maximum of 16.0% in Site 2. The total number of first sigth individuals found during the sampling period was 81 for Site 1, 373 for Site 2 and 147 for Site 3 (Figure 6). At Site 4, a major silting event was observed 3 months after AHU deployment and after 5 months all replicates were buried, so monitoring stopped from that point onwards. Overall repeat sightings accounted for 23.5% of the total observed seahorses.

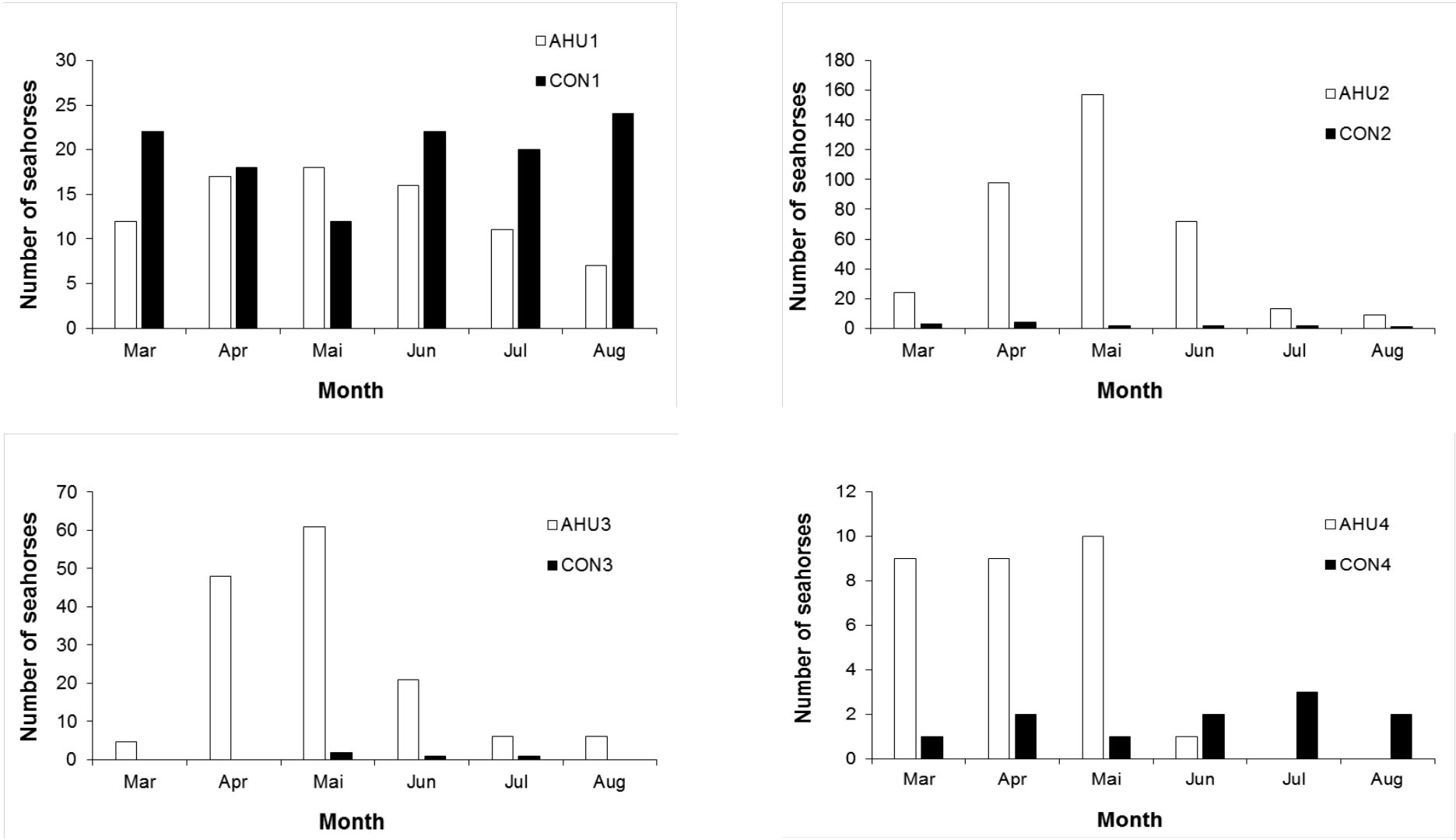

Figure 7.4: Overall number of monthly sighted seahorses on the AHU and Control sites (CON#) located at Site 1 to 4.

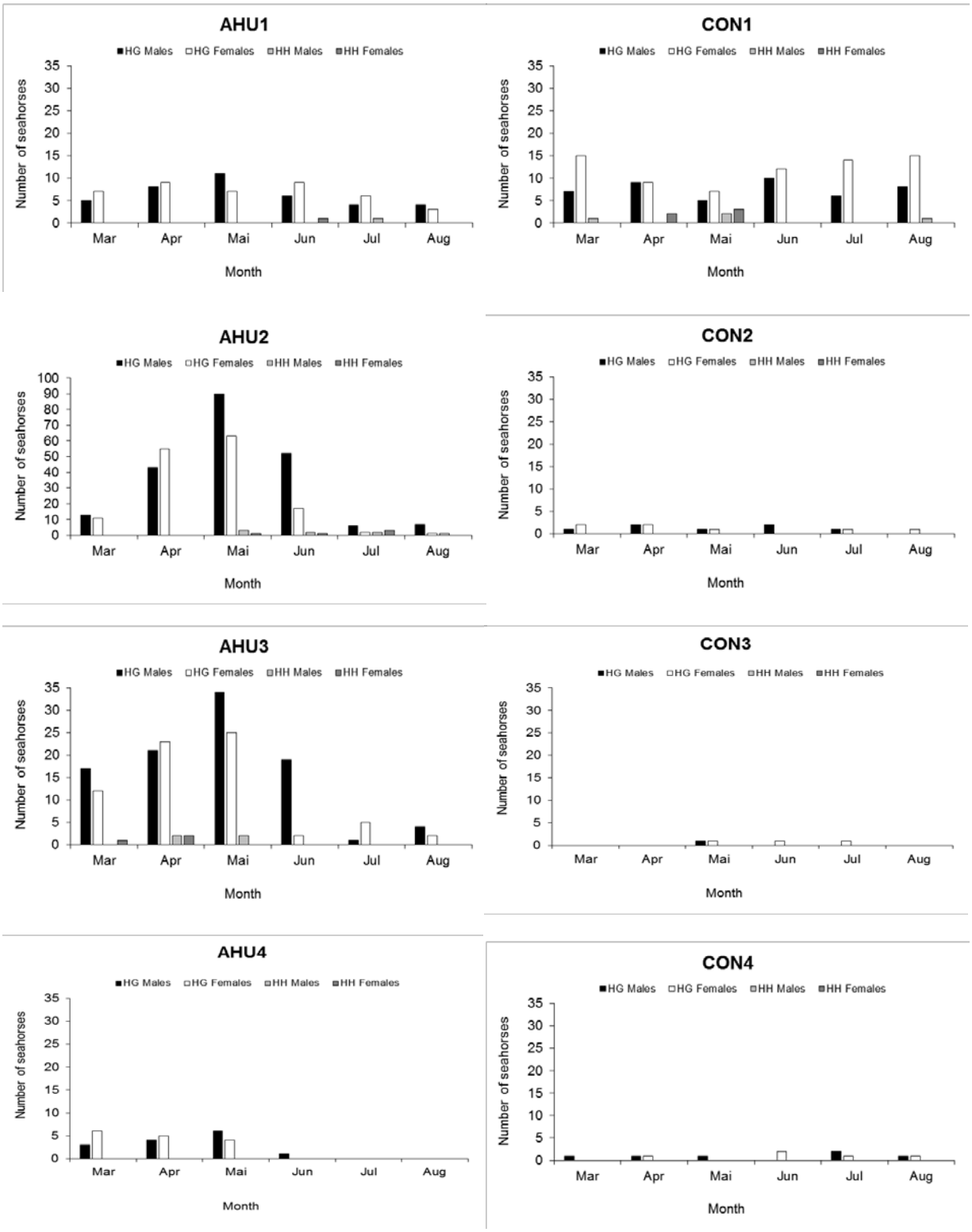

Figure 7.5: Total number of monthly sighted seahorses by gender and species on the AHU located at Site AHU1 to 4.

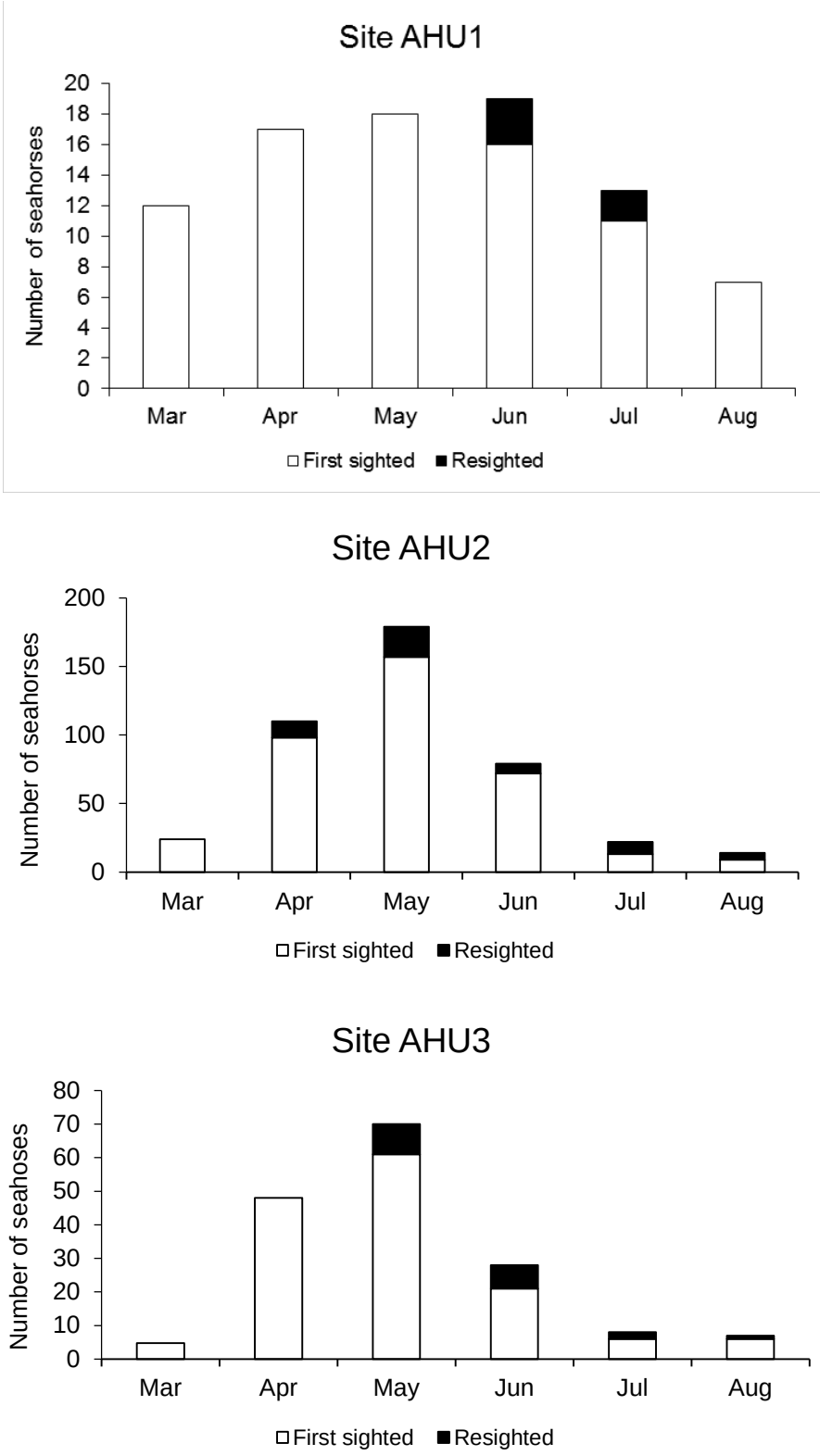

Figure 7.6: Resight history of *H. guttulatus* (□ – first sighted and ■ – re-sighted) for Site 1 to 3 on a monthly basis throughout the experiment

7.5 Discussion

In many countries, the use of artificial structures has been a recognized approach to compensate for dwindling natural ecosystems and has been used as an alternative habitat for various marine organisms (Sogard & Able, 1994; Kenyon *et al.*, 1999; Lee *et al.*, 2001; Shahbudin *et al.*, 2011). In particular, artificial seagrass units (ASUs) have been proven to be a valid replacement, even if temporary, while the natural habitat recovers (Bell *et al.*, 1985; Sogard, 1989). These structures have also proved adequate to provide a suitable habitat for small fishes, increase prey density, promote nursery grounds for juveniles, and to provide predatory protection for small fishes, thus playing a useful role in maintaining balance in marine environments when the natural habitat has been degraded or destroyed (Shahbudin *et al.*, 2011).

The use of artificial structures to rehabilitate damaged areas such as coral reefs, saltmarshes or other coastal areas, is still a subject of debate (Hauser *et al.*, 2006; Sirota & Hovel, 2006; Fernandez *et al.*, 2009; Vega Fernández *et al.*, 2009; Moberg *et al.*, 2011). Artificial structures have been used to reestablish fish populations as they provide shelter and feeding grounds for many communities (Ambrose & Anderson, 1990; Bohnsack *et al.*, 1994; Charbonnel *et al.*, 2002; Zalmon *et al.*, 2002; Claudet & Pelletier, 2004). On the contrary, many authors consider that they act as fish aggregation devices rather than increasing overall abundance (Grossman *et al.*, 1997; Pickering & Whitmarsh, 1997) and in the case of sedentary fish such as seahorses, these assemblages in a non-protected area might render them more vulnerable to adverse human related activities or natural events. Although these arguments may be true, the use of these structures has the potential to provide a long-term beneficial effect on the recovery of seahorse populations, particularly as one component of a wider rehabilitation and management plan. The use of these artificial structures may provide an improved habitat for seahorses, promoting the settlement of individuals that will provide more opportunity for reproduction and protection from predators. In the case of the Ria Formosa, seahorse aggregation on AHU should not affect their collection from the wild as there are no reports of ongoing fisheries that specifically target seahorses. However, such aggregations may render them vulnerable to damaging activities such as illegal bottom trawling and dredging.

Seahorses' cryptic behavior, poor swimming ability and their dependence on using their prehensile tail to grip holdfasts, makes them vulnerable to habitat loss or degradation (Curtis *et al.*, 2007) as they are equally dependent upon holdfast structures for hunting and predator avoidance (Foster & Vincent, 2004; Curtis & Vincent, 2005; 2006). Furthermore, habitat degradation may limit migration and diminish the re-pairing of eventual widowers or of a disrupted couple (Vincent & Sadler, 1995; Foster & Vincent, 2004; Vincent *et al.*, 2005). Habitat patchiness will result in a sparse distribution of seahorses and therefore may decrease the opportunities for sexual interaction thus contributing to a significant long-term population decrease. Caldwell & Vincent (2012) reported a 94% decrease in *H. guttulatus* abundance and a 73% decrease in *H. hippocampus* abundance over the course of the past 10 years in this same study area. The causes for this drastic reduction are currently under study, but it became evident that the diminished holdfast availability ranks high as a factor to consider (Chapter 2). In the Ria Formosa lagoon the seahorses dependence for holdfasts is also influenced by the fact that the Ria is a highly hydrodynamic, where the average maximum current speed can go up to 1.25 $m.s^{-1}$ (Pacheco *et al.*, 2010), a constraint that enhances the need for stable holdfasts in order to prevent them to be swept away from their preferred habitats (Curtis & Vincent, 2005). Previous studies suggest that when exposed to different water flows, *H. guttulatus* not only prefer current speed below 1.0 $m.s^{-1}$ (Chapter 6), but also prefer also more stable holdfasts which they grasp close to the base to minimize instability.

Despite their relatively higher abundance, *H. guttulatus* has been reported to favor more complex habitats (Chapter 2) when compared to *H. hippocampus*, using both biological and artificial holdfasts (Curtis & Vincent 2005). *H. guttulatus* is the most abundant seahorse species in the Ria Formosa, with the established population to be one order of magnitude greater than *H. hippocampus* (Curtis & Vincent, 2005). This fact was confirmed in this study as the *H. guttulatus* was found at higher densities in all AHUs and in all surveyed sites. In fact, the presence of *H. hippocampus* on the AHU was almost negligible, considering all structures deployed. This could be attributed to the fact that *H. hippocampus* are not attracted to these structures due to their lower demand for complex habitats, generally occur at lower abundance, or, more concerning, if these low numbers are an expression of the continuous population decrease first reported by Caldwell & Vincent (2012). In addition, in this study,

H. hippocampus has been reported up to 20 times less abundant than the other sympatric species (Chapter 2).

Seahorses benefit from macroalgae *Codium* sp. (Correia, pers. obs.) as holdfasts, as the size of this seaweed provide not only more stable holdfast than other macroalgae and seagrasses, but can also help decrease bottom currents, thus creating a beneficial habitat for small prey and the species that depend on them. The previous results obtained by Correia *et al* (2013) (Chapter 6) showed that the best compromise for artificial holdfasts are those that mimic *Codium* sp.. Those findings conducted *ex situ*, are now confirmed by the present study performed in natural conditions, as these structures were readily colonized by seahorses at higher densities than the surrounding areas of less holdfast availability. In addition, it was also observed during the surveys that all AHUs were colonized by many other fish species, including other syngnathids (pipefishes), different species of gobies, wrasses and sparids, invertebrates (including cephalopods) and crustaceans, including small Caridae shrimps, mysids and amphipods that constitute the base of seahorse diet. Therefore, the use of these AHUs generates the potential to recreate complex habitats which benefit not only seahorses but also a number of other species. It was also observed that the artificial materials used to build the AHUs maintain their effectiveness for at least 6 months, a time period long enough to generate the potential to modify the deployed area, creating the conditions for seahorse settlement.

In this study, the highest seahorse densities were observed in AHUs deployed in the vicinities of the locations with low habitat complexity (Site 2) and barren area (Site 3). This might indicate that the AHUs work as an aggregation device, and provide a viable habitat for seahorses. While assemblages of seahorses in a non-protected area may render them more vulnerable to adverse human related or natural events, this aggregation might also contribute to an increase in opportunities for sexual interaction in depleted populations and so to help to support population recovery. In fact, the artificial structures used have the ability to provide habitat for seahorses at high densities of at least up to 13.1 seahorse m^{-2}. This density is far greater the 0.51 seahorse m^{-2} reported by Curtis & Vincent (2005) and the 0.04 seahorse m^{-2} reported by Caldwell & Vincent (2012). The high densities observed on the AHU also confirms that the patchy distribution of seahorses, in particular *H. guttulatus* (Curtis &

Vincent, 2005), might be due to holdfast availability. In fact, seahorses were observed at lower densities on the AHU deployed close to high complexity habitats (Site AHU1). In this case, seahorses seem to maintain their preference for the natural available holdfasts. Overall, these results suggest that these AHU have the potential to aggregate seahorses in damaged habitats, but when in presence of natural complex habitats, seahorses tend to remain in their natural habitats. Sites with remaining natural complex habitats are unlikely to be part of a habitat or species recovery plan where AHUs might be considered as a management option, while our findings here suggest that degraded sites might benefit from AHUs to aid recovery as part of a management plan.

Seasonal variation in seahorse densities was observed in all AHU during the course of the experiment. Trends of decline were observed between the months of May until August in both trials in consecutive years. This reflects the same patterns observed in the monitoring of seahorses in the Ria Formosa over the course of a year (Chapter 4). In addition, bryozoan (*Zoobotryon verticillatum*) thrived and covered the entire AHU area, from August to October 2012 for the Prototype and from June to August 2013, in all sites. This event might be also responsible for the seahorse density decrease observed at that time, but again the seasonal increase of this bryozoan has been recorded in previous years (author's observation). Bryozoan progressively covered the entire AHU making it difficult for seahorses to grasp the AHU holdfast and so reducing the effectiveness of those artificial structures.

Based on the photo-identification used on *H. guttulatus* seahorses, only a low percentage of repeat sightings was reported when compared with those reported by Correia *et al.* (2014) at sites without AHUs, i.e. 23% versus 38%. Additionally, the area occupied by the AHU was within the home range of $20m^2$ reported by Curtis & Vincent (2006) and is far smaller than the maximum distance moved by *H. guttulatus* of 150 m reported by Caldwell & Vincent (2013). These facts might suggest that, although the artificial structures provide alternative habitat for seahorses, these fish might prefer other nearby areas with more suitable characteristics, using the AHU as a temporary dwelling area. This information can be checked either by using molecular markers, as microsatellites, in order to understand where the seahorses are moving from and eventually recruiting (either nearby areas or longer distances), or by short term tagging studies (Caldwell & Vincent, 2013).

In sum, the AHU design and methodology used in this study proved to be useful tools for habitat enrichment, especially in areas with low holdfast availability. These structures could be used to recover damaged habitats, even temporarily, contributing to the improvement of seahorse populations in similar scenarios. Our results suggest that holdfast availability is an important factor to consider and influences the seahorse density in the Ria Formosa lagoon. The results from this experiment can be foreseen as a future guideline for the use of artificial structures to improve degraded and impacted habitats within the Ria Formosa and even in other similar situations, as part of a management plan.

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Chapter 8. General Discussion

The main goal of this thesis was to identify the possible causes for the significant decline in both seahorse species reported by Caldwell & Vincent (2012) and suggest tools that could mitigate this negative trend.

8.1 Long-term monitoring

The latest seahorse surveys in the Ria Formosa identified continued fluctuations in populations of long-snouted seahorse, *H. guttulatus* and short-snouted seahorse, *H. hippocampus* abundance over the past decade (Chapter 2). Although Caldwell & Vincent (2012) reported a dramatic decrease for *H. guttulatus* (94%) and *H. hippocampus* (73%), the findings from this study demonstrated that seahorse population abundance appears to be increasing. In fact, *H. hippocampus* mean density observed in this study was higher than those reported in 2001-2002 for the same 16 sites. *H. guttulatus* population seems to be recovering but still under the abundances reported in 2001-2002. *H. guttulatus* was positively correlated with holdfast availability, being the only variable with statistical significance as a predictor for this species' abundance, which agrees with Curtis & Vincent (2005). This fact stresses the importance of holdfast availability for seahorse settlement. In order to overcome the gaps in the seahorse population surveys, spatial and temporal distribution of the wild populations of *H. guttulatus* and *H. hippocampus* should be assessed on an annual basis and ideally combined with wider habitat surveys of the seagrass beds within the Ria Formosa. Due to the seasonal variation reported in this study, short term surveys (less than one year period) may result in biased information (Chapter 4), as only long term surveys are able to identify the environmental variables that influences the seahorse population dynamics.

8.2 Underwater Visual Census

Considering the low mobility, small home range and patchy distribution of the seahorse species, the UVC methods used to assess their density needs to be appropriate to the species and characteristics of the site as reported for several other species (De Girolamo & Mazzoldi, 2001; Harvey *et al.*, 2004; Colton & Swearer, 2010). Our results showed that higher seahorse densities were found when using the UVC method developed in this study, but only for one of the two seahorse species found in the study site, i.e., *H. guttulatus*. This may have been due to the patchy distribution reported for this species (Curtis & Vincent, 2006) related to habitat constraints such as low holdfast availability. Considering the observed fluctuations in seahorse populations within the Ria Formosa lagoon in the last 10 years (Curtis & Vincent, 2005; Caldwell & Vincent, 2012), we conclude that the UVC method here proposed is more appropriate for a long-term monitoring programme at this site as it was shown to cope better with low density population scenarios. The UVC method designed in this thesis could be affect by skews when laying the transect tape, due to water current effects and/or surveyor's inexperience. This might lead to minor displacements of the survey area. This could probably be solved by delimiting the bottom area with a tape or rope, thus marking the research area. Although this could be possible in low current and minor human activities areas, this does not seem to be viable in the Ria Formosa. Strong water currents up to 1m s^{-1} make it very difficult for a tape to be maintained at one spot without being damaged or destroyed. In addition, silting events that commonly occur could bury the markings and thus rendering this method unsuitable for this location.

8.3 Seasonal monitoring

Statistically, there was no correlation between environmental data and *H. hippocampus* density, a result that reduces the theoretical population predictability of this species. However, in this study, this might be influenced by the low abundance recorded during the surveys, and notably their absence in several survey occasions. *H. guttulatus* abundance decreased from May to July and then increased from August to December. This decrease in abundance occured during the species' breeding season, when *H. guttulatus* and *H.*

hippocampus pregnant males were observed (from May to late August in agreement with Lourie et al. (2004) suggesting that the search for mate could be the driver. The highest *H. guttulatus* density (0.51 seahorse m^{-2}), reported in November 2012 at Site 1 was similar to the highest density reported in 2002 by Curtis & Vincent (2005). Nevertheless, lower seahorse densities were observed at the same site in different months (0.05 seahorse m^{-2} in May 2012). This fact highlights the importance of an extended monitoring effort in order to avoid bias due to population density seasonal fluctuation. Ideally, three surveys events should be performed during the year:

a) March to April - This should allow a more accurate estimation of the population, before the start of the breeding season. In these months the temperature is still low and seahorses should be less active, i.e., less dispersal.

b) May to June - This will provide information about the percentage of pregnant males that may contribute to the recruitment; Surveys performed in this period should bring more insight on reproductive success of the species.

c) October to November - This period is recommend to record the juveniles and therefore estimating the recruitment success;

In conclusion, this study suggests that seasonal events are responsible for population abundance fluctuations, particularly for sedentary species as seahorses that are more vulnerable to environmental stressors.

8.4 Photo-identification

The results obtained in both the preliminary *ex situ* and *in situ* experiments fully supported the applicability of the photo-identification as an individual non-invasive marking technique to be used in long-snouted seahorse, *H. guttulatus*, populations. Overall, roughly one third (31.3±13.1%) of all photographed animals were resighted. This result suggests a sedentary behavior of this species, when compared with other migrant fish species (Templeman, 1984; McGovern *et al.*, 2005), and agrees with their well-documented small home range (Curtis & Vincent, 2005; 2006; Caldwell *et al.*, 2011). Several animals were resighted in multiple

occasions throughout the year up to 9 out of 11 monthly surveys. Although Curtis & Vincent (2006) reported that home-range for this species after settlement was approximately 20m, *H. guttulatus* have a patchy distribution that may be conditioned by the level of habitat complexity and population density. In this study, the preliminary experiment proved that photo-identification is an efficient methodology to successfully identify juvenile seahorses ageing from 6 month old onward. In younger individuals kept in captivity, spot patterns are already present as it starts to manifest at 3 months of age (Jorge Palma, pers. obs.). Nevertheless, the spots are still too small and difficult to distinguish. In conclusion, photo-identification is a useful tool for mark-recapture studies and can be successfully used to survey population abundance of *H. guttulatus* in consecutive years and may be fully applicable to other *Hippocampus* species with characteristic con-specific morphological traits. Photo-identification has proven to be a valid method for seahorses aged six months old or older. Nevertheless, this method should be tested at younger ages, as the spot patterns may be visible at that time. Also, visual comparison should be assessed as it could lead to a quicker identification of each individual, as the use of the software Manta I3SM is highly time consuming. Visual comparison has already been used and validated as individual identification in other syngnathid species (Monteiro *et al.*, 2013). Photo-identification should be tested in different seahorse species as a capture-recapture tool, as it is a less invasive method when compared to VIFE.

8.5 Artificial Holdfast Units – in laboratory

As *H. guttulatus* favors more complex habitats than *H. hippocampus*, and were naturally found using both biological and artificial holdfasts (Curtis & Vincent 2005), this species was used to test the potential of artificial holdfast units (AHUs). Results show no individual or sex related differences between holdfast preferences, demonstrating a clear preference for one particular AHU type. Although it was expected that *H. guttulatus* would prefer the S1 holdfast, as it mimics the seagrass bed, this study showed that this species have a significant preference for the holdfast that mimics *Codium sp.* macrolgae. As for holdfast density, seahorses showed preference for densities of 156 and 256 holdfasts.m^{-2}. Habitat patchiness will result in a sparse distribution of seahorses and therefore may decrease the opportunities

for sexual interaction and so contributing to a significant long term population decrease. The use of these artificial structures may provide an improved habitat both for *H. guttulatus* and *H. hippocampus*, promoting the populations settlement and ultimately maximizing both individual and sexual interaction. This study provided the first step in establishing how AHUs might be used as a management tool for enhancing declining seahorse populations in the Ria Formosa lagoon.

8.6 Artificial Holdfast Units – *in situ*

Artificial structures have been used to reestablish fish populations as they provide shelter and feeding grounds for many communities (Ambrose & Anderson, 1990; Bohnsack *et al.*, 1994; Charbonnel *et al.*, 2002; Zalmon *et al.*, 2002; Claudet & Pelletier, 2004). On the contrary, many authors consider that they act as fish aggregation devices rather than increasing overall abundance (Grossman *et al.*, 1997; Pickering & Whitmarsh, 1997). Although these arguments may be true, the use of these structures has the potential to provide a long term beneficial effect on the recovery of seahorse populations, particularly as a component of a wider rehabilitation and management plan. The use of these AHUs generates the potential to recreate complex habitats which benefit not only seahorses but also a significant number of other vertebrate and invertebrate species. It was also observed that the artificial materials used to build the AHUs maintain their effectiveness for at least 6 months, a time period long enough to generate the potential to modify the deployed area, creating the conditions for seahorse settlement. The highest seahorse densities were observed in AHUs deployed in the vicinities of the locations with low habitat complexity (Site 2) and barren area (Site 3). This might indicate that the AHUs work as an aggregation device, and provide a viable habitat for seahorses. In fact, the artificial structures used have the ability to provide habitat for seahorses at high densities of at least up to 13.1 seahorse m^{-2}. This density is far greater than the 0.51 seahorse m^{-2} reported by Curtis & Vincent (2005) and the 0.04 seahorse m^{-2} reported by Caldwell & Vincent (2012). The AHU design and methodology used in this study proved to be useful tools for habitat enrichment, especially in areas with low holdfast availability. These structures could be used to recover damaged habitats, even temporarily, contributing

to the improvement of seahorse populations in similar scenarios. Artificial structures have shown to be a valid method for habitat recovery. In the summer, the AHU have a higher probability of being colonized by benthic sessile animals which indicates that these structures can create habitat not only for seahorses but for other species as well. The deployed AHU only covered a small bottom area of 12 m^2 per site and still had promising results regarding seahorse colonization. Increasing the area covered by these artificial structures might increase their effectiveness as habitat recovery tool, particularly in degraded habitats. Furthermore, the deployment of these artificial structures needs to be carefully planned. The location should be evaluated first, for example, areas close to inlets will be prone to silting events and might bury the AHU, as observed in Site 4 (Chapter 6). Also, human related activities might contribute for the destruction or removal of some AHU, like boat anchoring and fishing (particularly jigging fisheries).

8.7 Final considerations

These are the major findings of this study:

- Seahorse populations in the Ria Formosa have shown evidence of recovery in recent years, but remain depleted compared to a decade ago, particularly for *H. guttulatus*.

- The importance of ongoing population monitoring was identified, and we recommend that it should be done at least annually. The months for the monitoring should be selected according to the research goal, i.e., March/ April - to assess population status; May/June - to understand reproductive activity; October/November - to study the recruitment success;

- Holdfast availability was identified as the major factor influencing seahorse populations, particularly for *H. guttulatus*.

- The UVC method developed in this study is better suited to low abundance species like seahorses, and should be the method of choice in future studies. This method has applicability to other benthic and/or sedentary species.

- There is considerable variation in abundance of seahorses within the course of a year, in response to seasonal events. This needs to be considered in the design of monitoring programmes.

- Further variables should be investigated that could influence population fluctuations e.g. food availability, sound, habitat limitation for the newly settled juveniles and predation.

- Photo-identification was proven to be a useful tool for individual seahorse (*H. guttulatus*) identification applicable for both *in situ* and *ex situ* surveys. This tool has the potential to work on other species, providing that they have distinctive skin patterns.

- We developed a viable AHU design that was proven to be effective for seahorses *ex situ* and *in situ*. The application of this method needs to take careful consideration of site and status of local seahorse populations. In cases of very low abundance, the aggregating influence of the AHU may be beneficial in increasing the potential of reproductive events.

- MPAs offer a means of protecting key sites and are recommended for the focal sites of high abundance identified in this study, particularly for Site 3 (Chapter 2) and Site 1, Site 3, Site 4 (Chapter 4).

8.8 Future research

There is still research to be done towards identifying the factors that lead to seahorse population decline or that drive abundance fluctuations. It is of utmost importance to continue annual monitoring focal sites within the Ria Formosa to assess habitat changes and potential implications on seahorse abundance. Although a recovery seems to be occurring, considering the high numbers of juveniles sighted during 2012, it is vital to continue surveys to follow the trend of the seahorse populations during an extended time frame. However, extensive monitoring programs require the financial support of either a Research Project or by private companies, as well as enough manpower to complete these tasks. In previous seahorse populations monitoring programs in the Ria Formosa (Curtis & Vincent, 2005; Caldwell & Vincent, 2012), as well as the current study, many volunteers participated in the data collection. In fact, engaging the participation of young researchers in the field work can

provide a good trade-off between them and the research institutions. The Centro de Ciências do Mar (CCMar) of Universidade do Algarve has the necessary tools to promote this synergy, and has been recognized as a leading research Centre in the field of marine biology and ecology in Portugal. In addition, new tools have been made available to relate recreational diving and scientific research, through 'citizens in science'. *iSeahorse* (http://www.iseahorse.org) was recently created by Project Seahorse and collaborators have an online based tool to participate and report seahorse sightings and more recently, trends. This tool has the premise that anyone can report seahorse sightings, adding information such as location, species, photos and other data. This information is then reviewed and filtered by expert researchers. This tool will contribute to engage community of citizen scientists and conservationists with a common goal: to protect and increase our scientific knowledge on seahorses.

Another way to tackle the problematic of the added pressure to the seahorse population due to habitat lost and human related activities is through the implementation of Marine Protected Areas (MPA). This tool has proven to be very effective to protect sedentary fish from human related activities such as fisheries, boat traffic and recreational activities. In fact, one of our selected survey site (Site 5 *in* Chapter 4, 7) became protected due to a major silting event as local authorities limited the boat traffic and fisheries in that area due to safety issues because the main channels became too shallows (even in high tide) to navigate. After this intervention, we observed an increase in seahorse abundance in our monthly surveys (in 2010), with the highest density reported when compared to the remaining sites. Although this could be a preliminary indication of the value of protecting an area to promote seahorse settlement, it could not be confirmed over an extended period of time, as a new silting event occurred (in 2012), and led to a massive sand deposition, significantly transforming that habitat and forcing seahorse dispersal. Although the Ria Formosa is a protected area (Natural Park), it still allows fisheries and other recreational activities. Coordination between researchers and local authorities could allow the application of an MPA in targeted areas within the Ria Formosa. Due to their small home range behavior (Curtis & Vincent, 2005), seahorses do not need extensive areas to establish resilient populations, therefore small MPA's could be implemented in focal areas with little or no impact to the local fishermen communities. Moreover, as these areas would not only benefit seahorses, they could be regarded as small

recovery pockets for other commercially important species as well. However further research should be done in order to assess the impact of human activities in the local seahorse populations. Stressors related to water sports and intense boat traffic boat traffic, which dramatically increase the waterborne noises, can become a source of acoustic stress having a negative impact on the seahorse welfare (Anderson *et al.*, 2011). These authors reported an increased incidence of physiological disorders in *H. zosterae* when exposed to low frequency boat motor noise, suggesting that seahorses are prone to effects of surrounding noise. Our observations suggest the same problem, with *H. guttulatus* and therefore, further analyses are needed. There is still a gap in the knowledge of the life cycle of the seahorses in the Ria Formosa. After birth, the newly-born have a pelagic stage of 2 to 3 weeks. At this stage, the seahorses are vulnerable to predation and feeding success. In fact, newly born seahorses lack nutrient reserves which makes them readily dependent of prey availability. Failing to eat properly in the first few days will lead to physiological constrains such as over-inflation of the gas bladder, and ultimately, death (Palma *et al.*, 2014). There is very scarse information available about the habitat preferences of the newly-settled juveniles. In fact no seahorse smaller than 5cm were found during UVC in previous studies (Curtis & Vincent, 2005; Caldwell & Vincent, 2012), and only a few were found in this study (total of 6 seahorses). Identifying the habitat that seahorses at this stage choose is vital in order to pinpoint areas that should be protected.

New hypotheses have to be considered in order to explain the population variations that occur periodically. Food limitation, either for juveniles or adults seahorses, can be an important aspect to be considered, so further research as to be done in this regard. Natural fluctuations in prey abundance may have not only a direct impact in adult's condition, limiting the brood size and quality (Palma *et al.*, 2012), but also in juvenile survival, affecting overall recruitment. In fact, Palma *et al.* (2008) improved the condition of wild seahorses in captivity by more than 50%, feeding them with frozen prey *ad libitum.* This fact suggests that food availability might be an important variable to consider when addressing the causes that drive seahorse abundance. Information regarding condition index (height/weight) of the wild seahorse population should be investigated, using RNA:DNA ratio in case of the newly born/juveniles (Buckley *et al.*, 1999; Kono *et al.*, 2003; Smith & Buckley, 2003; Chicharo & Chicharo, 2008) and/or by weighing and measuring the seahorses (adults) found during UVC.

Ongoing research with zooplankton in the Ria Formosa could also shed some light in seahorse abundance fluctuations by comparing the natural seasonal fluctuations of that prey's abundance.

Climate change has been an increasingly addressed topic and has been recognized as having an important impact in species abundance (Klyashtorin, 1998; Henderson & Seaby, 2005; Ujvari *et al.*, 2010; Chessman, 2013). Changes in temperature might drive to seahorse dispersal in search for more suitable habitats. In fact, Aurélio *et al.* (2013) reported that increased temperature (up to 28ºC) might lead to metabolic depression in new born *H. guttulatus*, and consequently death. However adults showed a resilience to heat stress and are not expected to go through any physiological impairment and behavioral change. Although this information highlights the resilience capacity of *H. guttulatus* to high temperatures, this species prefers optimal temperatures of 20ºC (Palma *et al.*, 2008). This may support the hypothesis of seahorse dispersal to locations with more suitable environmental conditions, as the depth gradients in the Ria Formosa, due to its topography, create gradients in temperature (Correia, pers. obs.). Caldwell & Vincent (2013) reported that *H. guttulatus* may have broader home ranges, with movement up to 150m, in contrast to the 20 m^2 home range reported in previous studies (Curtis & Vincent, 2005). This new finding needs to be addressed in detail in order to better understand the movement behavior of seahorses and its implication in habitat occupancy and reproduction success. Acoustic tags have been used to study seahorse movements (Caldwell *et al.*, 2011; Caldwell & Vincent, 2013) but it is still in the early stages of development. This tool is quite expensive and, although it has been proven to have no effect on the tagged seahorse's health, it might diminish its cryptic capability, making it more vulnerable to predation and impair its predation success. Also, additional information can be added by using molecular markers, as microsatellites for multi locus genotyping, in order to better understand the seahorse home ranges and recruitment patterns. This will enable identification of new recruits in the study area and thus improving the population estimates. This method depends on live tissue collection from wild seahorses. Currently the tissue from the dorsal is widely used for these studies. However even the small collection of fin tissue, although having been proved to have no direct effect on seahorse mortality (Woodall *et al.*, 2012), can impair the proper locomotion of seahorses that rely on the dorsal fin as main means of movement (Consi *et al.*,

2001; Ashley-Ross, 2002). As alternative, skin filaments could be used in these studies. This tissue is less prone to have any negative effect on seahorse health. In fact, our laboratory trials have proved it to be a reliable option and with no apparent effect on seahorse health. Although both *H. guttulatus* and *H. hippocampus* may have skin filaments, it is more commonly observed in *H. guttulatus* (Correia, pers. obs.) so this species may be selected as case study in validating this tool.

Raising local awareness is a key factor in promoting better practices that can minimize pressure on the seahorse populations in the long run. During this thesis, several presentations were made in order to bring awareness to the public about the conservation actions that need to be taken in order to protect the seahorse populations in the Ria Formosa and their habitat. Seahorses have a great potential to be used as flagship species as they are very charismatic. This fact as allowed us to publicize our work through the local and international media. We participated in several interviews for the main Portuguese television networks (RTP1, RTP2, SIC, TVI) as well as radio (Antena 1, RUA, Radio Renascença) and magazines (National Geographic – Portugal in March 2011). In addition, we participated in international stories ("L'Algarve, côte enchantresse" – Arte; "An der Algarve" – MareTv). We also held an international symposium, "The Second Syngnathid Biology International Symposium" in Faro in 25-28 March, 2013. This allowed us to gather all the experts in the field and present and debate ongoing studies and future research.

The findings of this thesis provide valuable insights into the status and trends of the two seahorse species found in the Ria Formosa lagoon. New methods of survey and identification were developed that are a valuable contribution to seahorse research globally. This research also developed valuable tools to mitigate against seahorse population decline and help prompt recovery. We will look to develop a short management briefing from this research which can be integrated into conservation management plans for the Ria Formosa and its rich marine biodiversity.

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